Skeletal Muscle Adaptations Across the Surgical Timeline: Evidence for Prehabilitation to Mitigate Mitochondrial Dysfunction and Inflammation in Hepatopancreatobilairy and Colorectal Cancer Surgery Patients.

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ABSTRACT

Background

Prehabilitation programs aim to improve surgical outcomes and prevent comorbidities, however, the underlying mechanisms remain poorly established. It is established that surgery induces significant physiological stress on the body, therefore this study presents novel findings investigating skeletal muscle molecular adaptations across a surgical timeline whilst investigating the potential benefits of a prehabilitation programme in patients undergoing elective Hepatopancreatobilairy and Colorectal cancer surgery.

Methods

Twenty-eight patients (17 standard, 11 prehabilitation) provided three muscle biopsies from the vastus lateralis at key timepoints: baseline, pre-surgery and post-surgery. Patients in the prehabilitation group received HIIT training for a duration of two to four weeks prior to surgery and the comparator group received standard care. Western blotting analysis was completed to investigate changes in anabolic signalling, inflammation markers, and mitochondrial markers. An exploratory analysis on 13 inflammation cytokines was also completed using a LEGENDplex Multiplex bead-based assay.

Results

Western blotting revealed in the standard care group there was significant surgery-induced degradation of the mitochondrial electron transport complexes ($p<0.05$) that was not present in the prehabilitation group. IL-6 was significantly lower post-surgery in both cohorts (standard care: $p=0.004$, prehabilitation: $p=0.027$) reflecting the removal of a source of inflammation through tumour resection. FoxO3a was also significantly higher in the prehabilitation group following surgery indicating enhanced stress response signalling ($p=0.03$). An exploratory analysis using the multiplex assay highlighted significant differences for multiple cytokines in both care groups.

Conclusion

This study presents novel findings regarding surgeries impact of mitochondrial disfunction in skeletal muscle. The findings suggest prehabilitation may protect against surgery-induced skeletal muscle decline through preserving mitochondrial function. This study provides molecular insights into the potential benefits of prehabilitation on a cancer cohort and provides a platform for larger clinical studies to build on these preliminary molecular findings.

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Declaration

Data Statement

All the data presented within this thesis were collected, analysed and presented by me unless otherwise stated below.

Joel Lambert, at East Lancs teaching Hospital conducted the randomised control trial and collected the biopsies, however, all analyses on the skeletal muscle and presented herein were completed by me.

I declare that all the data presented is my own work unless stated otherwise, in this thesis was constructed by me. Appropriate referencing has been used for all the published literature referred to within this thesis.

None of the data presented within this thesis has previously been submitted for assessment towards a higher degree.

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Conflict of interest statement

No conflict of interests was declared.

1. Literature Review

1.0 Introduction

Cancer is a global health challenge which is responsible for one in six deaths worldwide (Bray et al. 2024). Cancer Surgery has been ongoing for thousands of years with the earliest recorded descriptions dating back to the ancient Egyptians (3000-2500 BC) (Faguet 2015). To this day, surgery remains the primary treatment modality for solid malignancies, offering potential curative outcomes for patients with localised diseases, therefore, offering the potential of complete recovery through removal of the tumour (Debela et al. 2021). However, surgery is frequently linked with severe physiological stress, immunosuppression- through a variety of metabolic and endocrine responses, and a variety of other consequences that can affect both short- and long-term prognosis (Finnerty et al., 2013; Hogan et al., 2011). Hepatobiliary (HPB) and colorectal (CR) cancer surgery has severe morbidity and mortality risks, with specific challenges posed by the intricacy of resecting tumours in these locations, as well as the severe stress effects of surgical trauma (O'Connell & Hoti, 2024). The liver, pancreas, gall bladder, and bile ducts (HPB system) are notorious for having intricate, complex anatomical relationships, especially through interconnected vascular supplies, this combination, alongside the location of the liver pancreas and bile ducts being so close to major blood vessels, makes HPB surgery considered one of the most complex and technically demanding types of cancer surgery (Gunasekaran et al., 2021). Similarly in CR cancer surgery, the associated high risk of infection, blood clots, bleeding, and a reduction in physical ability can all have a significant impact on one- and five-year survival rates (Giacomantonio and Temple 2000). With cancer incidences rising globally, observed healthcare outcomes have become a heavily relied on tool to assess the quality of medical care; with mortality, morbidity, and post-operative complications serving as objective indicators to evaluate central performance (Addae et al., 2017; Wijma et al., 2023).

Perioperative complications can be categorised into systemic and local complications; examples of systemic complications include:

- **Cardiopulmonary Complications:** Postoperative pneumonia, pulmonary embolism, and cardiac arrhythmias. These complications are

common because of the severe physiological stress of surgery, with patients with pre-existing conditions such as COPD and asthma being particularly vulnerable (Choi et al., 2023).

- **Sepsis and superimposed infection:** Despite being a rare complication of surgery (0.5-3%), post operative wound and superimposed infections can lead to sepsis, requiring prolonged hospitalization and intensive care (Zabaglo et al., 2024).
- **Venous Thromboembolic Events:** Deep vein thrombosis (DVT) and pulmonary embolisms are heightened risks due to prolonged immobility associated with surgery and imbalance between clotting and bleeding rates (Römer et al., 2024).
- **Nutritional Deficits and Anaemia:** Cancer patients suffer from a heightened risk of malnutrition, which can impair wound healing and muscle regeneration post-surgery, and can in some cases lead to anaemia from iron and vitamin deficiencies. Anaemia has been shown to predispose other complications in the perioperative period potentially leading to longer hospital stays, worsened wound healing (Pan et al., 2022).

Examples of local complications in HPB and CR surgery include:

- **HPB Surgery:** as alluded to earlier, HPB surgery has high peri-operative risk of bile leaks, anastomotic leaks, and liver disfunction due to the complexity of the liver, pancreas, and bile ducts (Kneuertz et al., 2012).
- **Colorectal Surgery:** Anastomotic leaks and bowel disfunction (including ileus and obstruction) and bleeding are frequent due to the nature of gastrointestinal surgery and the microbiome's role in healing. Surgical site infection is the most prevalent peri-operative complication in CR surgical populations and has severe consequences on patient recovery (pain, readmission, length of stay and sepsis) (Al-Amry et al. 2024).

Given these risks, optimising preoperative health levels and physiological resilience through preventative interventions, like prehabilitation, is becoming an increasingly relevant and highly used strategy to improve surgical outcomes and

reduce the risk of morbidities and mortality (Del Bianco et al. 2024; Lambert et al. 2024).

1.1 Prehabilitation in general and the associated role prehabilitation has in cancer surgery

Prior to surgery there is an opportunistic window for prehabilitation, defined loosely in this scenario as interventions to enhance physiological reserves in patients before undergoing the stress of surgery. This is typically prescribed through a combination of exercise, nutritional interventions and psychological support (Dunne et al. 2016; Molenaar et al. 2022). Research into prehabilitation has drastically increased since the 2000s, however, there is still variation in how beneficial prehabilitation is (Punnoose et al. 2023). Some evidence suggests prehabilitation can enhance preoperative outcomes and increase the patients' physical, nutritional, and mental status (Molenaar et al. 2019), as well as suggested to reduce perioperative complications in various cancer populations (Cho et al., 2025). However, research is still needed to understand the effectiveness across different types of cancer surgery. Due to the absence of standardised protocols and the variation amongst prehabilitation programmes it is difficult to compare studies and fully understand how valuable prehabilitation is (Guerra-Londono et al., 2024). This highlights the continued need for targeted research across all cancer types, and cares to develop effective, evidence-based programmes which could potentially be implemented into the National Health Service' (NHS) surgical pathways.

Cardiorespiratory fitness is one physiological parameter which has emerged to be a consistently important predictor of surgical outcomes across all populations. Patients with higher cardiorespiratory fitness alongside healthier skeletal muscle mass may have better surgical outcomes, primarily through increasing physiological reserve and withstanding the metabolic stress and major physiological demands of oncological surgery (Shrestha et al., 2025). However, while the clinical associations are well-established, the underlying biological mechanisms underlying these outcomes, and how different prehabilitation programmes influence these biological mechanisms, remain poorly understood (Rose et al. 2022; Blackwell et al. 2023; Shanmugasundaram Prema et al. 2025).

1.2. The Role of Skeletal Muscle in Cancer Surgery and Recovery

Research has started to report the role skeletal muscle and lean muscle mass play as a determinant of surgical outcomes, however, the pathophysiological mechanisms underlying are poorly established (Ansari et al. 2024). The role of sarcopenia (the loss of muscle mass and function in ageing) and cachexia (weakening and wasting due to chronic illness) (Hadzibegovic et al., 2020) have been strongly recognised for the increased risk of frailty, and are therefore highly associated with a plethora of negative pathological states and diseases, such as chronic kidney disease, peripheral artery disease, and some immunodeficiencies (Lee et al. 2015; Zhang et al. 2024). Literature findings support concepts that skeletal muscle plays a greater role than just movement, and acts also as a major metabolic reservoir, source of amino acids and as the primary site for glucose uptake and storage. These findings have therefore caused skeletal muscle to be appreciated as a potential predictor of systemic health and consequentially surgical recovery (Richardson et al. 1999; Bonaldo and Sandri 2013). Due to the extreme plasticity of skeletal muscle and the ability to continuously adapt to different stimuli (physical activity, nutrition and hormone signalling) changes in skeletal muscle health may have implications for systemic health. Institutions continue to study skeletal muscle due to the understanding that a reduction in muscle mass may impair post-operative recovery and reduce overall survival rates of oncology patients (Weerink et al. 2020; Tan et al. 2022). Therefore, this known plasticity of skeletal muscle provides a strong rationale to implement prehabilitation in the pre-operative period to maximise metabolic reserve, improve surgical recovery and reduce the economic burden associated with prolonged hospital stays and re-admissions (Hunter et al. 2019).

1.3. Mechanisms of Muscle Adaptation to Prehabilitation

To optimise prehabilitation outcomes, it is crucial to understand the molecular and cellular mechanism underlying the adaptations in skeletal muscle. While the clinical benefits of prehabilitation are increasingly well recognised, the biological pathways mediating these adaptations remain elusive. This section explores the key mechanism through which exercise interventions enhance skeletal muscle

physiology focusing on anabolic signalling, inflammatory modulation and mitochondrial adaptation.

1.3.1. Anabolic Signalling Pathways

Skeletal muscle mass and function are tightly regulated by anabolic and catabolic networks, with several key regulators being directly relevant to surgical recovery and prehabilitation outcomes. The interplay between protein synthesis and degradation determines muscle mass maintenance making the pathways crucial as prehabilitation targets (McCarthy and Esser 2010).

Mammalian target of Rapamycin (mTOR) serves as a central serine/threonine kinase that governs skeletal muscle mass by co-ordinating the different pathways. There are two biochemically and functionally distinct mTOR complexes (mTORC1 and mTORC2) responsible for the stimulation for protein synthesis whilst also acting to restrain catabolic processes (Yoon 2017).

Experimental models where mTOR has been downregulated demonstrate severe atrophy, suggesting it plays a crucial role as a regulator of muscle maintenance and as a therapeutic target (Sartori et al., 2021). In the context of prehabilitation, exercise induced mTOR activation represents a key mechanism through which resistance training programmes promote muscle protein synthesis and maintains or improves muscle mass. Downstream of mTOR is P70, which plays a pivotal role in stimulation of protein S6 phosphorylation, which enhances translation efficiency and protein synthesis (Artemenko et al. 2022). Its activation serves as a widely used indicator of mTORC1 activity (Hara et al., 1998). The phosphorylation plays a crucial role in the Akt-mTOR pathway and enhances translation efficiency and protein synthesis capacity enhancing protein synthesis rates allowing muscular hypertrophy (Meyuhas 2015; Saxton and Sabatini 2017).

AMP-Activated protein kinase (AMPK) is another central regulator of cellular energy status, becoming activated when the AMP:ATP ratio rises during metabolic stress (Yun and Zierath 2006). Once activated, AMPK promotes mitochondrial biogenesis and enhances oxidative metabolism to generate ATP whilst simultaneously inhibiting protein synthesis to limit energy consuming pathways (Trefts and Shaw 2021). AMPK maintains energy homeostasis and aids the adaptive response to exercise, which may make it a strong predictor of

energy stress within prehabilitation programmes. The co-ordinated activation of AMPK highlights metabolic flexibility and oxidative capacity suggesting an improved ability for the muscle to cope with the demands of surgery (Cantó et al. 2009). Outside of prehabilitation, the role of AMPK to create metabolic flexibility makes it a target for treating metabolic diseases like diabetes and obesity as AMPK plays a role in systematic, whole body energy homeostasis (Garcia and Shaw 2017)

The forkhead box O (FOXO) transcription factors, including FOXO1 and FOXO3, are key regulators of skeletal muscle protein turnover, particularly regarding catabolism. When under stressful conditions, such as metabolic stress or oxidative stress, FOXO translocates to the nucleus to promote the expression of muscle atrophy genes, for example MAFbx (Sanchez et al. 2014). FOXO activation also triggers autophagy, and mitophagy further activating catabolism in the skeletal muscle (Zhou et al. 2012). Through driving these protein degradation pathways, FOXO contributes to muscle degradation pathways, and the suppression of FOXO supports muscle maintenance and hypertrophy, making FOXOs an important mediator to understand how muscle responds to both prehabilitation and surgical stress (Liu et al., 2025).

The role exercise interventions have on modulating these signalling pathways is reasonably well established in healthy populations. To summarise, resistance and aerobic exercise stimulate mTOR and p70S6K activity, while suppressing FOXO-mediated catabolic signalling, thereby promoting protein synthesis and preserving muscle mass (Liu et al., 2025). Adequate protein intake provides the amino acid substrates necessary for muscle repair and growth (Morgan et al., 2023), highlighting prehabilitation is also dependent on nutritional strategies.
The understanding of molecular and metabolic adaptations allows interventions to be molecularly rationalised based on what is known to trigger changes in the body. Furthermore, specific prehabilitation protocols are critical for enhancing recovery and reducing perioperative complications in patients undergoing cancer surgery; Through understanding the mechanisms behind anabolic signalling, protocols can be designed to support muscle hypertrophy, improve functional capacity, metabolic resilience, and physiological reserve.

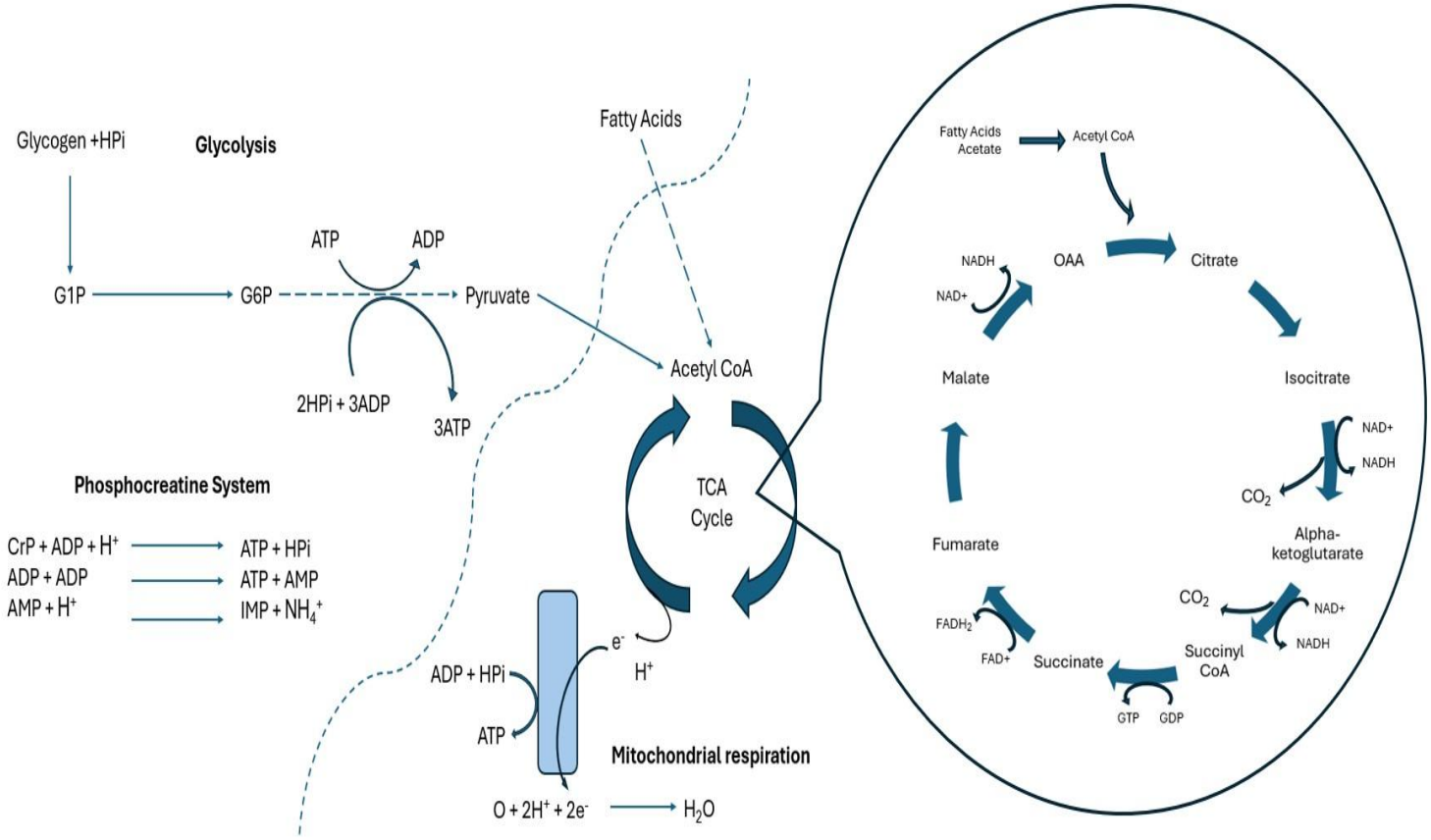
1.3.2 Inflammatory Markers and Muscle Response

Beyond anabolic signalling, skeletal muscle function is heavily influenced by inflammatory pathways which can accelerate catabolic processes and significantly impact surgical outcomes. Chronic low-grade inflammation is often present in older individuals (i.e. inflammaging (Sanada et al., 2018)) and in cancer patients and creates a hostile environment that promotes muscle wastage and impacts metabolic reserves (Cole et al., 2018). This is further heightened by cancer tumours secreting pro-inflammatory cytokines and chemokines that create a systematic catabolic state (Fairman et al. 2021; Setiawan et al. 2023). This tumour derived inflammation, combined with cachexia and the metabolic stress of surgery, present a poor environment for muscle maintenance and adaptations (Vaughan et al. 2012; Li et al. 2022).

Pro inflammatory cytokines such as IL-6 and TNF-a play central roles in muscle catabolism by activating NF-kB. This activation of NF-kB promotes the transcription of inflammatory genes and catabolic factors, similar as to how FOXO acts (Baker et al. 2011; Webster et al. 2019). Inflammation influences muscle metabolism through altering insulin sensitivity and glucose uptake. Chronic inflammation impairs insulin signalling through phosphorylation of insulin receptor substrates which reduces the muscle's capacity for glucose uptake (Inoue and Janini Gomes, 2024). As a result, the muscle's ability to serve as a metabolic reservoir through glycogen storage is compromised, leading to the potential of heightened physiological stress in surgery and recovery.

Exercise and nutritional interventions can reduce basal inflammation levels and prevent NF-kB activation (Scheffer and Latini, 2020), therefore, limiting the catabolic signalling and creating a favourable, anabolic environment. Nutritional strategies complement exercise prehabilitation programmes through adequate protein uptake, antioxidants, omega-3 fatty acids all support the reduction in oxidative stress and inflammatory signalling. The reduction in systemic inflammation through these interventions contributed directly to improved recovery trajectories and morbidities in patients undergoing cancer surgery (Liu and Chang 2018; Jameson et al. 2021).

1.3.3. Mitochondrial Enzymes and Changes



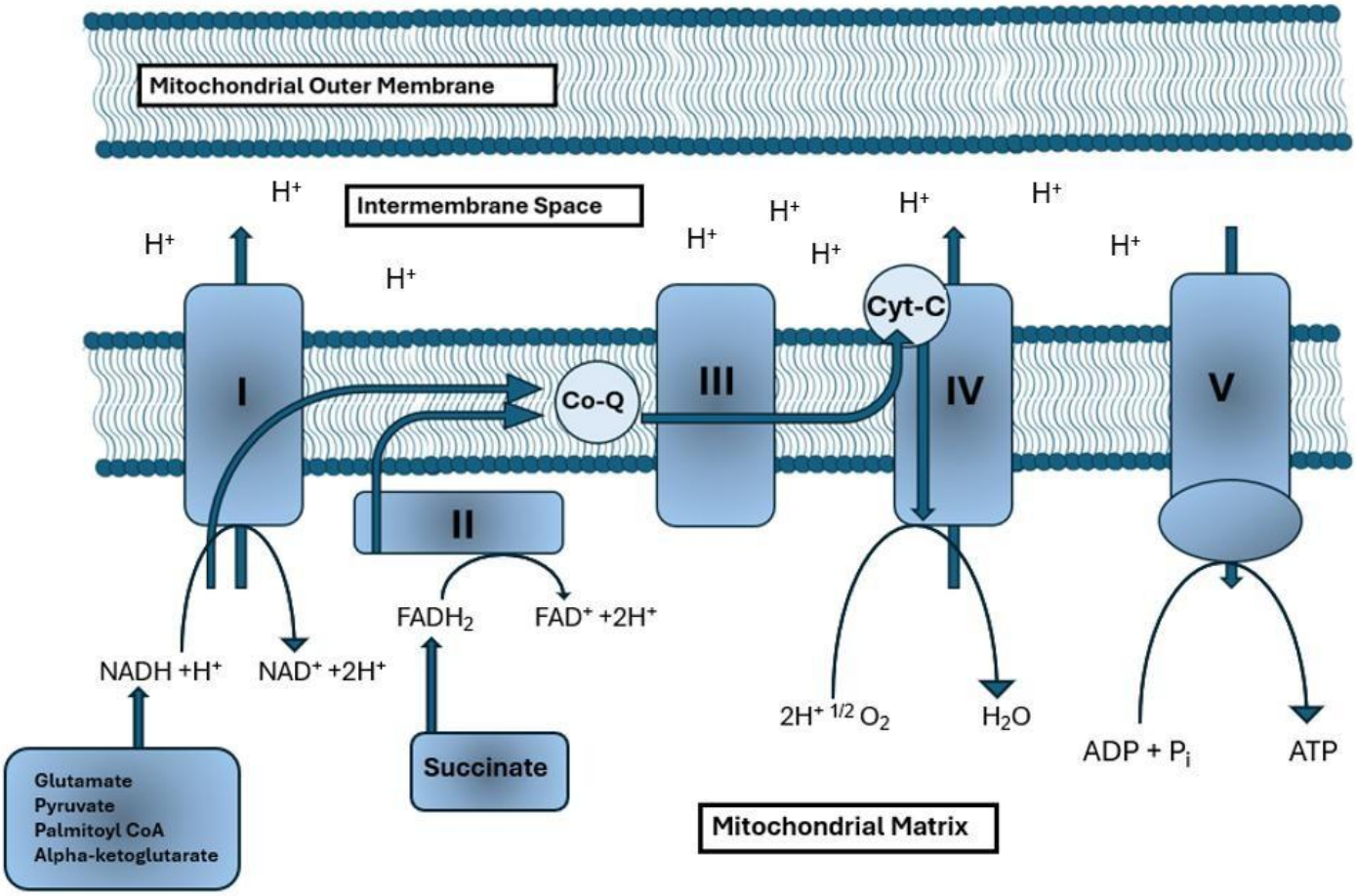
The known benefit of improved functional capacity and cardiovascular changes draws attention to mitochondria as a potential mapping tool to predict the impact of prehabilitation. Mitochondria are rod-shaped, membrane-bound organelles present in most eukaryotic cells. Colloquially known as the “powerhouse of the cell”, the primary function of mitochondria is as the primary energy generating organelles within skeletal muscle cells, through the generation of ATP for skeletal muscle contraction and metabolic function. As shown in Figure 1, the ATP-phosphocreatine system can only support ATP generation for around 1015 seconds and glycolysis for 2 minutes, metabolic energy requirements are mostly met through oxidative phosphorylation (Baker et al. 2010). While often simplified to this energy-production role, mitochondria are sophisticated organelles whose function extends far beyond basic ATP generation, encompassing roles in calcium homeostasis, reactive oxygen species management, and cellular signalling that are particularly relevant to surgical stress and recovery (Suomalainen and Nunnari 2024).

Figure 1- The three energy systems of muscle ATP regeneration, adapted from. The three energy systems of muscle ATP regeneration, adapted from (Baker et al. 2010).

The process of oxidative phosphorylation represents the most efficient pathway for cellular energy production. This multi-step process begins with the oxidation of acetyl-CoA in the citric acid cycle (Krebs cycle) through a series of redox reactions that generate the electron carriers NADH and $FADH_2$ (Ahmad et al. 2023).

These electron carriers then donate electrons into the electron transport chain, which is shown in Figure 2, where Complexes I and II facilitate the transfer of electrons through a series of protein complexes embedded in the inner mitochondrial membrane. The energy released from electron flow drives the active transport of hydrogen ions (Protons) across the inner mitochondrial membrane through Complexes I, III, and IV, hence creating an electrochemical gradient. This proton gradient provides the ability for ATP synthesis to turn ADP to ATP as protons flow back through Complex V through coupling proton flow to the phosphorylation of ADP (H. Liu et al., 2025).

Figure 2- The electron transport chain of the mitochondrion.



The efficiency and capacity of this oxidative system directly influence muscular endurance, fatigue resistance, and recovery capacity (Hendrickse et al., 2021). Patients with higher mitochondrial density and oxidative enzyme activity typically demonstrate superior exercise tolerance, faster recovery from physical stress, and enhanced metabolic flexibility, positioning mitochondrial function as a key determinant of surgical resilience (Befroy et al., 2008). However, it should be noted other determinants, including mitochondrial quality control mechanisms, muscle fibre type distribution, systemic inflammation, substrate availability, and overall metabolic health, also play important roles (Goodpaster and Sparks, 2017).

Prehabilitation interventions are designed to enhance oxidative capacity and metabolic efficiency to therefore improve functional capacity. These adaptations may manifest as increased expression of key oxidative enzymes, including citrate synthase, succinate dehydrogenase, and cytochrome c oxidase, which collectively enhance the muscle's capacity for aerobic energy production

(Fernandes et al. 2020; Lavin et al. 2022). Additionally, mitochondrial adaptations to exercise also include improved coupling efficiency between oxygen consumption and ATP production, enhanced calcium handling capacity, and increased antioxidant enzyme expression to manage exercise-induced oxidative stress (Vargas-Mendoza et al. 2021).

The clinical relevance of these mitochondrial adaptations extends beyond exercise capacity to encompass surgical recovery and complication prevention. Enhanced mitochondrial function provides greater physiological reserve to withstand the metabolic demands of surgical stress and recovery. Furthermore, improved mitochondrial respiratory capacity may enhance cardiopulmonary function (Knuiman et al., 2021), which reduces the risk of post-operative pulmonary complications which represents a significant source of morbidity in cancer surgery patients.

The integration of resistance and aerobic exercise within prehabilitation programmes creates complementary adaptations, with resistance training promoting muscle protein synthesis and hypertrophy, while aerobic training enhances mitochondrial density and oxidative capacity (McGlory et al., 2016). This dual approach optimises both the structural and metabolic components of muscle function, providing comprehensive preparation for the physiological challenges of cancer surgery and recovery (Bennett et al. 2022; Blackwell et al. 2023).

1.4. Methodologies for Muscle Biopsy Analysis

Having a greater understanding of the molecular mechanisms underlying muscle adaptation requires the ability to analyse cell culture models, animal models and human muscle biopsies. Direct analysis of human muscle biopsies provides mechanistic insights into how exercise prescription can impact populations in disease states through analysis of cellular pathways changes in regulation/expression of genes, proteins and transcription factors. A range of methodologies have been developed to investigate both structural and functional properties, all with their own advantages and limitations, highlighting the need for multimodal investigation using a range of analytical approaches to give comprehensive insights (Zschüntzsch et al., 2022). This section discusses a range of methodologies to investigate muscle adaptation mechanisms and

particularly focuses on western blotting and multiplex assays- the methodology used in the following clinical study.

1.4.1. Muscle Biopsy Collection

To allow for analytical techniques of skeletal muscle samples, it is crucial that there are appropriate collection, handling and processing steps in place as shown in Figure 3. Anaesthesia is used to numb the area before a Bergström needle is inserted, typically into the vastus lateralis, where a muscle biopsy can be removed and the wound can then be closed. Tissue is then snap frozen in liquid nitrogen and stored at -80ºC to prevent further protein degradation (Andrew Shanely et al., 2014).

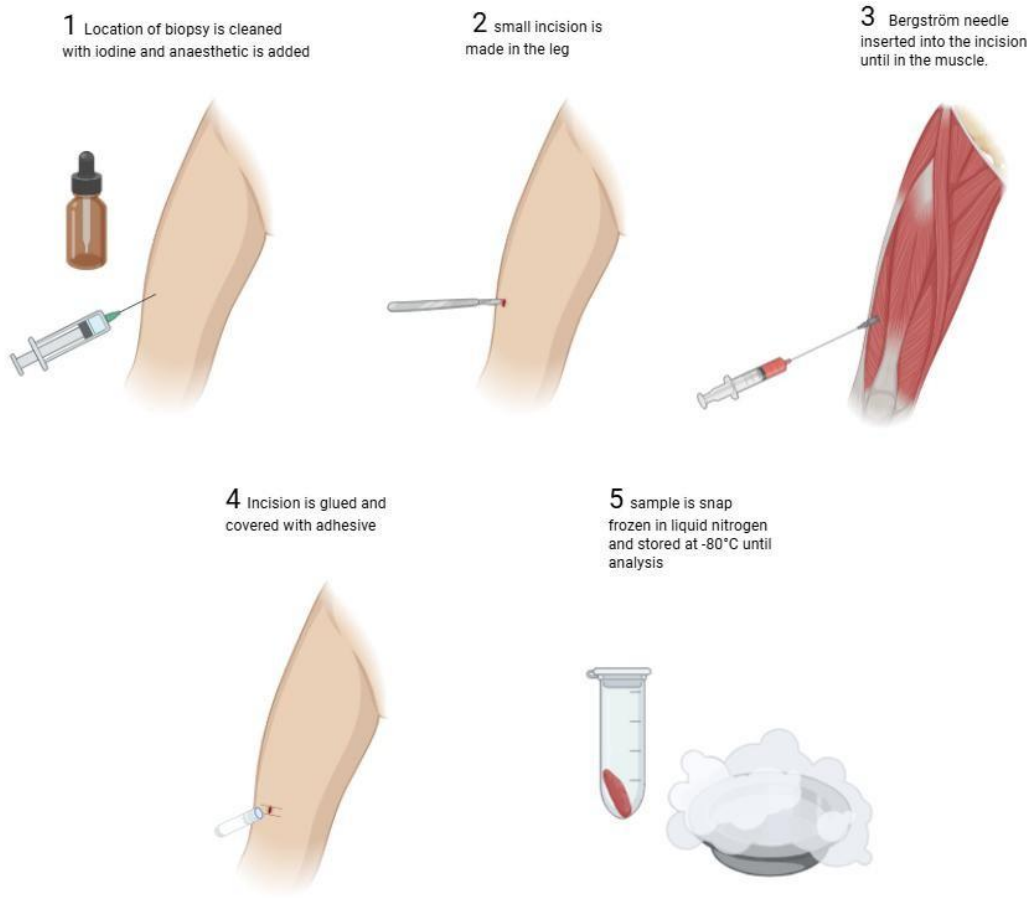


Figure 3- process of muscle biopsy sampling

1.4.2. Western Blotting

Western blotting is long regarded as the cornerstone for assessing protein abundance and post-translational modifications in skeletal muscle research (Galpin et al. 2012). Originally developed in the late 1970s and first dubbed "western blotting" in 1981, the fundamental principle underlying this method involves the following steps:

- Homogenisation of muscle tissue
- Separation by Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE)
- Transfer from the get to membrane through electrophoretic transfer
- the separation of proteins by molecular weight, before then transfer
- blocking of the membrane and probing with primary antibody
- secondary antibody probing

- detection through chemiluminescence or fluorescence imaging.

A full western blotting methodology and rationale behind the steps can be found in the methods section.

Western blotting is invaluable to clinical researchers trying to understand signalling pathways and molecular adaptations. In this scenario, western blotting allows the quantification of anabolic signalling components, inflammation markers, and mitochondrial enzymes- giving the ability to demonstrate changes throughout the surgical pathway (Bass et al., 2016).

Despite the widespread utility, western blotting does have inherent limitations which must be considered in experimental design and interpretation of data. Western blotting provides “semi-quantitative” rather than absolute quantification, as it provides a relative comparison of protein levels, but not an absolute measure. Antibody specificity is crucial as cross-reactivity and nonspecific binding can lead to false conclusions which may misinform clinical practice; Therefore, normalisation strategies are essential for ensuring robust conclusions. Western blotting is notoriously technically demanding with variability in outcomes potentially stemming from several sources therefore it is crucial that researchers maintain standardised protocol throughout experimentation (Murphy and Lamb, 2013). Despite the limitations, western blotting is an insightful tool which methods, such as proteomics, metabolomics, transcriptomics, and advanced imaging techniques (confocal microscopy, electron microscopy) can complement to provide multi-level mechanistic insights (Soderstrom et al., 2023).

1.4.3. Multiplex Assays

While western blotting allows targeted protein analysis and the ability to compare between groups, the investigation of inflammation and other complex biological complexes through multiplex assays can allow full quantitative measures and, in this case, the analysis of multiple analytes simultaneously- this is particularly valuable in muscle biopsy research as samples are finite and the ability to measure several cytokines and chemokines in a 50ul sample is valuable.

The LEGENDplex assay completed in this study, is a type of bead-based multiplex assay; meaning a flow cytometry-based assay where beads coated in a detectable fluorescence specific to different protein targets are captured by

antibodies conjugated to identifiable microspheres where beads are separated by size and internal fluorescence intensities (Jun et al., 2012). Each bead set is conjugated with a specific antibody and acts as “capture beads” for that specific analyte. Hence, when the capture beads are incubated with the sample, each analyte binds to the specific capture beads. Following an incubation period between the sample and the beads, flow cytometry analysis identifies specific bead populations through fluorescence intensity measurements- each bead population is distinguished by the fluorescence signature it is coated in allowing the detection of multiple analytes in as a single assay (Moncunill et al. 2014; Lehmann et al. 2017). The ability to assess multiple markers- in this case inflammatory chemokines and cytokines- in a single biopsy provides a comprehensive inflammatory panel that can give a clear picture of the pro and anti-inflammatory process ongoing through the prehabilitation programme and the surgical pathway (Liu et al., 2022; Zhang and An, 2007). Despite little research on human tissue, multiplex assays should become a favourable tool for researchers when investigating cytokine levels, due to the cost effectiveness, large datasets acquired and simple protocol.

1.4.4 Alternative Analytical Approaches for Skeletal Muscle

There are other beneficial analytical approaches which can aid an understanding of the molecular pathway and changes to protein expression in skeletal muscle. Histological analysis is the microscopic examination of tissue which provides essential information regarding the structural adaptations and basic muscle architecture including fibre size distribution, capillarisation and necrosis (Campbell and Maani 2023; Gurina and Simms 2023). Electron microscopy can be used to assess mitochondria morphology, size, density and cristae structure which may highlight subtle adaptations not apparent through other analytical approaches (Santhoshkumar and Narayanappa, 2022). There are also emerging areas in proteomics, metabolomics and transcriptomics being used to identify novel targets and pathways involved in muscle adaptation, as well as provide insights into gene expression changes and changes in pathway utilisation (Wilkinson et al., 2024).

1.5. Implications and Future Directions

Biopsy-based research has been instrumental in advancing our understanding of how prehabilitation modulates skeletal muscle biology at the molecular level (Smeuninx et al. 2021). Enhancing understanding of the molecular mechanisms underpinning prehabilitation programmes can allow for a greater understanding of specific personalised prehabilitation dependent on the cancer type, location, and demographic information (Shrestha et al. 2025). By stratifying patients according to baseline muscle health, mitochondrial capacity, and their inflammation profile, interventions can be tailored to optimise the timeframe before their surgical date. Future research should continue to aid understanding on mechanistic insights, while emerging technology may allow the integration of multi-omics that integrate transcriptomics, proteomics, metabolomics, and epigenetics data, to aid understanding and help create personalised strategies to optimise recovery by completely understanding the complexity of skeletal muscle. By targeting skeletal muscle as both a biomarker and a therapeutic pathway, prehabilitation offers a powerful opportunity to reduce morbidity, improve resilience, and ultimately enhance survival in surgical oncology populations. The understanding of key molecular targets may also allow pharmacological interventions to be integrated into prehabilitation to further enhance surgical outcomes.

1.6. Conclusion

Cancer surgery, particularly HPB and CR patients, represent an essential, yet high risk intervention. With the recognition of skeletal muscle as a key determinant to patient outcomes, the importance of optimising metabolic health, reducing inflammation, and enhancing mitochondrial function has become apparent. The literature has begun to demonstrate the benefits of prehabilitation interventions in optimising pre-surgical physiological state. Recent research has highlighted the understanding that exercise prescription is beneficial regarding mitochondrial capacity, inflammation, and anabolic signalling in healthy populations (Blackwell et al., 2023); however, less research has been completed in ageing, clinical populations. Looking forward, the field of exercise medicine and oncology is beginning to gauge an understanding on how mechanistic insights can guide patient specific outcomes, allowing the benefits of

prehabilitation to be further understood. The goal of prehabilitation research extends beyond pre-operative improvements and encompasses both peri-operative complications, morbidities and 1- and 5-year survival rates. Through not only aiding scientific understanding, prehabilitation research provides practical pathways to aiding the economic status of the NHS, with the over-arching goal being the integration of prehabilitation programmes into surgical pathways.

2. Aims and objectives

This thesis aims to investigate the impact of a structured prehabilitation program on skeletal muscle in patients undergoing elective HPB and colorectal cancer surgery.

Aims and Objectives

The specific aims of this thesis were:

1. To Investigate if prehabilitation could preserve or enhance mitochondrial function in skeletal muscle compared to standard care throughout the surgery timeline.
2. To Investigate whether prehabilitation could affect inflammation markers and anabolic signalling in skeletal muscle compared to standard throughout the surgery timeline.
3. To Investigate whether changes in mitochondrial function correlated with cardiopulmonary exercise testing data.
4. To Investigate if prehabilitation could link measurable protective effects against surgery-induced muscle metabolic decline.

The specific objectives of this research are to:

1. Compare changes in skeletal muscle mitochondrial electron transport complexes and proteins involved in mitochondrial dynamics, inflammation markers, and anabolic signalling markers baseline, pre- and post-surgery between patients undergoing prehabilitation versus standard care.
2. Assess whether prehabilitation modifies systemic inflammatory profiles, using multiplex cytokine analysis.

3. Explore associations between changes in mitochondrial function, and cardiopulmonary exercise testing (CPET) metrics such as anaerobic threshold and $\dot{V}O_{2peak}$
4. Provide evidence on the potential benefits of prehabilitation for preserving muscle metabolic health throughout the surgery timeline.

3 Clinical study

3.1 Introduction

Despite advances in surgical techniques since the early 2000s, HPB and CR cancer surgery continues to provide unique challenges and remains a difficult and physiologically demanding operation. This is further heightened by the older age population typically undergoing treatment, with high complication rates and comorbidities (up to 50% in some centres), attention has turned to optimising the pre-operative state of patient to reduce the physiological burden imposed through these cancer surgeries. With the incidence of cancer continuing to rise globally, surgical outcomes are used to assess the quality of medical care, with mortality, morbidity, and post-operative complications serving as objective indicators of healthcare provider performance. Research conducted prior to this study has highlighted that pre-operative fitness states can serve as a strong predictor of surgical outcomes (Billé et al., 2021), therefore, maximising the preoperative state prior to cancer surgery has come to the forefront of attention for healthcare professionals (Mcgladrigan et al., 2025).

Prehabilitation is loosely defined as interventions to enhance the physiological reserve of patients before the stress of surgery (Santa Mina et al., 2015). It can include a combination of exercise, nutritional interventions and psychological support that evidence suggests can enhance functional capacity and reduce perioperative complications in various cancer populations (Lambert et al., 2024). Cardiorespiratory fitness, skeletal muscle mass and clinical outcomes have been shown to increase following multimodal prehabilitation (Dunne et al. 2016b; Minnella et al. 2017; Bousquet-Dion et al. 2018; Janssen et al. 2019; Carli et al. 2020). Current studies have highlighted that the ability to enhance surgical recovery is a key motivator for patients to complete prehabilitation protocols (Powell et al., 2023). It is well established that improvements in

cardiovascular fitness are associated with improved post-operative outcomes (West et al. 2014; Santa Mina et al. 2015), through increasing physiological reserve and preventing frailty. However, the biological mechanisms by which prehabilitation may manifest these clinical benefits remain poorly understood. With muscle being a major metabolic reservoir, playing a key role in systematic homeostasis and stress response, the relationship between muscle health and surgical outcomes has become of central interest, however the exact mechanisms and implications of changes to muscle health and prehabilitation remains elusive.

Mitochondrial networks are particularly of interest due to the known relationship between prehabilitation and CPET outcomes, specifically beneficial changes to anaerobic threshold. Through the surgical timeline, mitochondria face extensive stress due to the increased demands of cellular ATP whilst simultaneously under excessive stress from inflammatory mediators and oxidative stress which may impair the function of mitochondria to function properly, therefore by analysing skeletal muscle and mitochondrial proteins, we can start to understand how interventions impact mitochondrial health and therefore metabolic capacity (Go et al., 2018; Hill and Van Remmen, 2014).

Enhancing understanding of the molecular mechanisms underpinning prehabilitation programmes can allow for a greater understanding of specific personalised prehabilitation based off the cancer type, location and demographic information (Włodarczyk, 2025).

Whilst exercise programmes have been shown to induce changes in skeletal muscle in cancer populations (Blackwell et al., 2023), to our knowledge this is the first trial to address HPB and CR cancer patients. This therefore address the critical knowledge gap regarding a severely high-risk cancer population where the surgical outcomes are notoriously poor. The focus on muscle biopsy analysis highlights an area of prehabilitation which is typically less studied due to the issues gathering muscle biopsies, therefore this study presents novel findings whether structure pre-operative exercise interventions can preserve mitochondrial function, aid in reducing pro-inflammatory cytokines and maintain anabolic signalling when under severe physiological stress (Robergs et al., 2004).

3.2 Methods

3.2.1 Ethical Approval

The analyses presented in this paper form part of the randomised clinical trial (SPECS Study, NCT04880772). National Health Service (NHS) ethical approval was received from the Leeds East Research Ethics Committee (approval number: 21/YH/0069). Concurrent approval from the Health Research Authority (HRA) was also gained (IRAS number 290723). The study Protocol was preregistered and published on ClinicalTrials.gov (NCT04880772).

3.2.2 Patient Recruitment

This clinical trial was a between groups randomised controlled trial (prehabilitation versus standard care). Patients were recruited from and tested at East Lancashire Hospitals Trust, a tertiary centre covering a 1.8 million patient population in Northwest England, UK.

From July 2021 to October 2023 a pool of potential patients was received from the respective weekly multi-disciplinary team (MDT) meetings. All patients undergoing resection for colorectal cancer (CRC) and liver resection for colorectal liver metastases (CRLM) and hepatocellular carcinoma (HCC) were assessed against eligibility criteria. All patients recruited were deemed suitably fit with an American Society of Anaesthesiologist (ASA) score of 1-3 (Ferrari et al. 2021). Written informed consent was gained prior to enrolment and randomisation and all testing conformed to the Declaration of Helsinki (7th Revision) and Good Clinical Practice. Patients were randomised to either the standard care group (SG) or prehabilitation group (PG) with a ratio of 1:1 using a computerised block randomisation method on the ‘sealed envelope’ online platform (sealedenvelope.com). A member of the research team performed the randomisation after cardiopulmonary exercise testing (CPET). The randomisation sequence was automatically date and time stamped and verified after the last patient was recruited.

3.2.3 Study Conduct

All assessments were done at three-time points: (i) baseline, (ii) preoperative, and (iii) post-operative. ‘Baseline’ was defined as the period of the first

assessment once enrolled on the study. ‘Preoperative’ covered a period one to three days before surgery and hence was after the period of prehabilitation. ‘Post-operative’ was defined as the recovery period (6-16 weeks) after surgery.

3.2.4 Prehabilitation Exercise Programme

A remotely supervised prehabilitation exercise programme was delivered by a qualified personal trainer. The exercise regimen involved 40-minute sessions of aerobic and strengthening exercise conducted 3d.wk^{-1} for 2-4 weeks depending on the date of surgery. Each session involved 5 mins warm up, then 20 mins at 60-70 % peak oxygen consumption ($\dot{V}O_2$ peak) followed by 5 min recovery period. Following this, strengthening exercises focussing on core muscle groups were performed using 5kg resistance bands. Alternative and equivalent exercises were employed by the personal trainer based on patient mobility factors and level of ability (training programme can be found in the appendix).

One session per week was monitored via a video platform by the personal trainer. Prehabilitation group (PG) participants were given a video of the exercises performed by the personal trainer to be done independently for the other two sessions per week. All patients in the PG were given an exercise log to fill in which was verified at the preoperative visit. All the PG had bespoke advice from a surgical dietician certified by the Health and Care Professions Council. Prehab patients received a multivitamin Forceval (Alliance Pharmaceuticals Limited, Chippenham, United Kingdom) for 30 days. Further details of our prehabilitation protocol are available open access at www.surgicalbridges.co.uk/SPECS.

3.2.5 Standard Care Group

The standard care group were given generic information about their treatment pathway by their cancer care teams. No specific nutrition recommendations were given. Patients were provided with the opportunity to attend surgery school, but this was not mandatory. Surgery school was a service designed to provide information on all aspects of perioperative care including lifestyle modification such as smoking cessation and reduction in alcohol consumption.
This service was also available to the Prehabilitation group.

3.2.6 Demographic Data Collection

Demographic factors including age, sex, body mass index (BMI), radiological diagnosis, chemotherapy treatment, Clavien-Dindo complications, hospital length of stay (LoS) and 90-day mortality were recorded on a purpose-built secure database.

3.2.7 Skeletal Muscle Biopsies

Skeletal muscle biopsies were taken from the vastus lateralis using a Bergström needle under local anaesthesia (Andrew Shanely et al., 2014). Biopsies were immediately snap frozen in liquid nitrogen and stored at -80°C until analysis was completed.

3.2.8 Western Blotting Protocol

Day 1

Homogenisation of the tissue

Tissue was removed from -80°C freezer and dissected free of fat and connective tissue on a petri dish before being chopped finely using a scalpel blade to increase the efficiency of homogenisation (Goldberg, 2008). Tissue was homogenised with a glass pestle and mortar in 1ml of Tris lysis buffer (50 mm Tris, 1 mm EDTA, pH 7.5) supplemented with a protease inhibitor (Sigma Aldrich; USA) on ice for 30 seconds, The protease inhibitor cocktail was added to prevent degradation of target proteins and preserves post-translational modifications. The sample was then spun using a centrifuge (Fisher Scientific, USA) for 15 minutes at 10,000RCF at 4°C. The supernatant was then collected with the remaining fraction then discarded; this step is completed to ensure a clean supernatant for downstream analysis by isolating soluble proteins and molecules by removing the debris (Dounce et al. 1955; Taylor et al. 2013; Bass et al. 2017).

Quantification of the tissue and sample preparation

Protein concentration was then quantified using a nanodrop spectrophotometer (Thermo Scientific, USA) which measures absorbance at 280nm and estimates concentration based off the assumption that 1A (280) unit corresponds to approximately 1mg/ml of protein Coefficient of variation was calculated by reading each samples concentration 3 times, all samples presented CV of under 10%. This method of quantification was chosen as it requires very little sample (0.5-2ul) compared to colorimetric assays, and samples were finite (Desjardins et al., 2010). Samples were then all diluted to the same concentration of 0.67µg/µl to load 10ug per well as per Taylor et al. (2013):

Volume (µL) = Desired protein amount (µg)/ Protein concentration (µg/µL)

15 (µl) = 10 µg/ Desired Concentration

Desired Concentration = 10/15 = 0.67 µg/µl

The samples were then made up into 1ml stock solutions through dissolving with 4x Laemmli buffer (250ml Laemmli containing10% BME, 750ml sample). Laemmli (Bio-Rad, USA) is a sample buffer containing SDS detergent which is used to unfold proteins and coat them in a negative charge, eliminating the shape and charge differences allowing the protein to be separated purely by size during electrophoresis. Beta-mercaptoethanol (BME) (Bio-Rad, USA) was also added 10% to break disulphide bonds and fully linearise proteins. A small hole was poked into each Eppendorf tube and heated to 95°C for 5 minutes. This is to help fully unfold the proteins and ensure tertiary structures are unfolded, as well as improving BME activity (Creighton, 1988; Laemmli, 1970).

Electrophoresis

Electrophoresis was used to separate out denatured and negatively charged proteins- due to the fractional resistance of a protein as it passes through micropores in the gel and as a result the proteins are separated by their molecular weight via an electrical current (Ornstein, 1964). Both sides of a BioRad criterion western running tank were filled with Bio-Rad XT mops running buffer (Bio-Rad, USA) A 26 well Bio-Rad XT Bis-Tris 4-12% gel (Bio-Rad, USA) was added into each side of the tank (Bio-Rad, USA). Precast gels were used

due to the longer shelf life and ability to store better. The trough in each of the gel was filled with XT mops running buffer, before the removal of the green combs from each gel, ensuring the wells remain intact. 3ul of Bio-Rad kaleidoscopic protein ladder (Bio-Rad, USA) was loaded at both ends of both gels (Bass et al., 2017). 15ul of the diluted samples were loaded left to right with the 2 central wells left empty. The lid was placed on the tank and electrodes connected to the power pack (Bio-Rad, USA) and the gel was ran for 1hr at 180V or until blue tracking dye ran off the bottom, whichever occurs first, for proteins of a low molecular weight), the electrophoresis was stopped before the tracking dye ran off the bottom. Electrophoresis was run at a constant voltage due to the linear relationship between protein migration and voltage. Bands are formed by the inability of proteins to continue to pass through smaller pores as they are too large, therefore they cluster forming stacks.

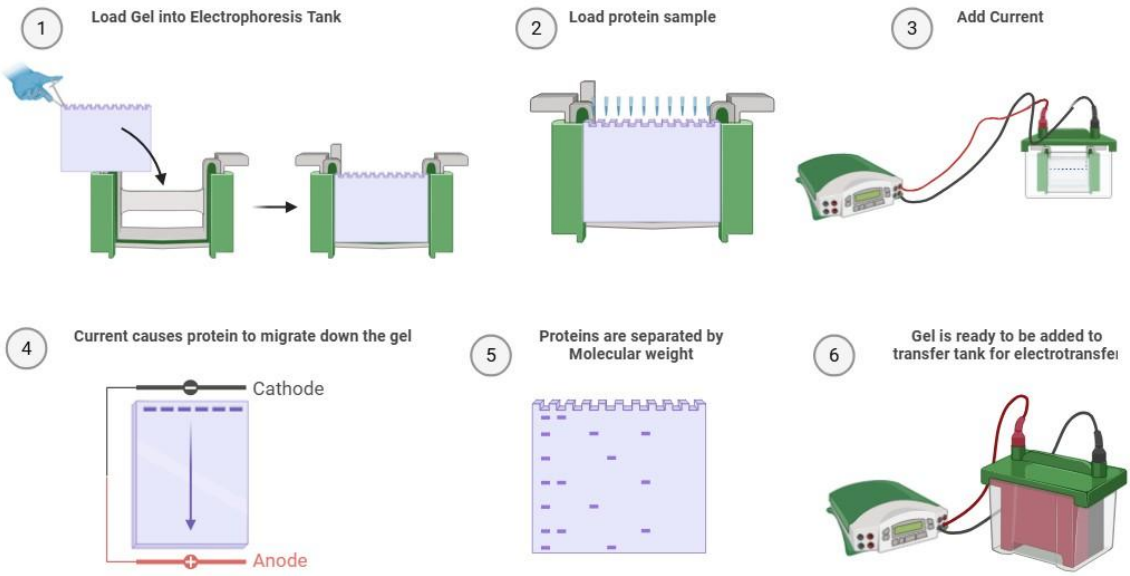


Figure 4- Electrophoresis steps in Western Blotting

Transfer to membrane

Following electrophoresis, the proteins were immobilised onto a Polyvinylidene fluoride (PVDF) membrane (Bio-Rad, USA) through electro transfer. Through moving the negatively charged proteins from the cathode to the anode when a lateral electrical current is applied, the proteins became bound to the

membrane. 2litres of transfer buffer (Cell Signalling Technology, USA) were prepared prior to electrophoresis using 20% methanol, with 1L in freezer and 1L in 4°C cold room. Following electrophoresis PVDF membrane was cut into the appropriate size for the gel. PVDF membrane was activated/permeabilised by wetting in 100% methanol for 1 minute before being washed with transfer buffer. Membrane was only ever handled with tweezers and never allowed to dry. After electrophoresis the gel cassette was removed from the running tank (Bio-Rad, USA), and the cassette is cracked using a cassette opening lever. The front plate is removed and is rinsed with double distilled water (ddH_2O) to remove any excess SDS as this interferes with the binding of proteins to PVDF and causes faster protein migration through the membrane. The gel was then placed face down in transfer buffer for 5-10mins (Mansfield, 1995). To remove the gel from the cassette ddH_2O was used to pry the gel away from the cassette taking care not to tear the gel. The pre chilled transfer buffer was then removed from the freezer and added to the Bio-Rad transfer tank. The freezer block was then added to the back of the tank. The gel sandwich is then prepared on the black side of casing plate for transfer in the order as follows:

- Black casing plate
- Black fibre pad
- Blotting paper
- Gel
- PVDF membrane.
- Blotting paper
- Black fibre pad.
- Red casing plate
- Close plate using clasp

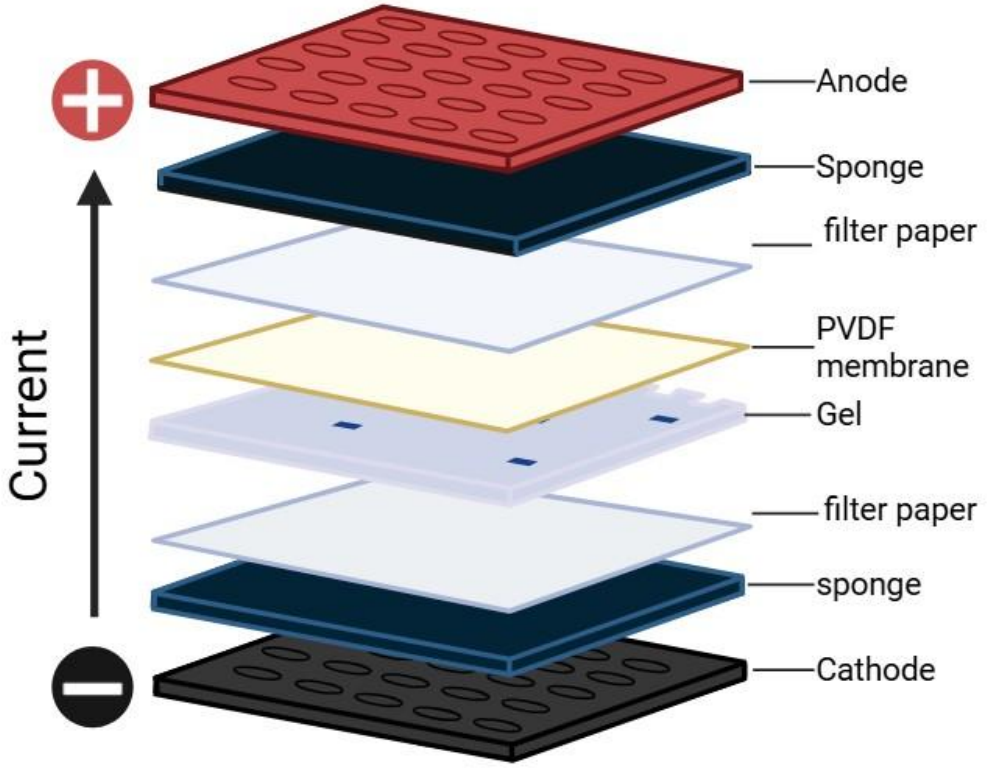


Figure 5- Cassette gel sandwich stack assembly order for electro-transfer

It is crucial to avoid air bubbles between PVDF membrane and gel, this was accomplished by using a roller over the back of the PVDF membrane and on top of the filter paper once placed over (Alegria-Schaffer et al., 2009). PVDF membrane can be marked as to which side is the front of the membrane, and which samples are loaded were. The gel sandwich was then placed into transfer tank ensuring the black side faces the cathode and red side faces the anode. The transfer was then run overnight at 20V in a cold room at 4°C (Burnette, 1981).

DAY 2

Confirmation of transfer

Once transfer was completed overnight, the gel sandwich was disassembled. The membrane was briefly placed in Tris buffered Saline with Tween-20 (TBST) (Cell Signalling Technology, USA) as excess sodium dodecyl Sulfate (SDS) can affect binding of the primary antibody. To determine protein transfer efficiency

and equal loading, the PVDF membrane is placed in a dish and Ponceau S stain (Thermo Scientific, USA) was added. The membrane was placed on a shaker at 100RPM until pink protein bands start to appear. Whilst the membrane is stained the PVDF membrane is cut in half along the empty two middle bands. The membrane was then removed from ponceau and placed in 1x TBST, before being washed several times in 1x TBST until pink tinge is removed from membrane (~15mins) (Sander et al., 2019)

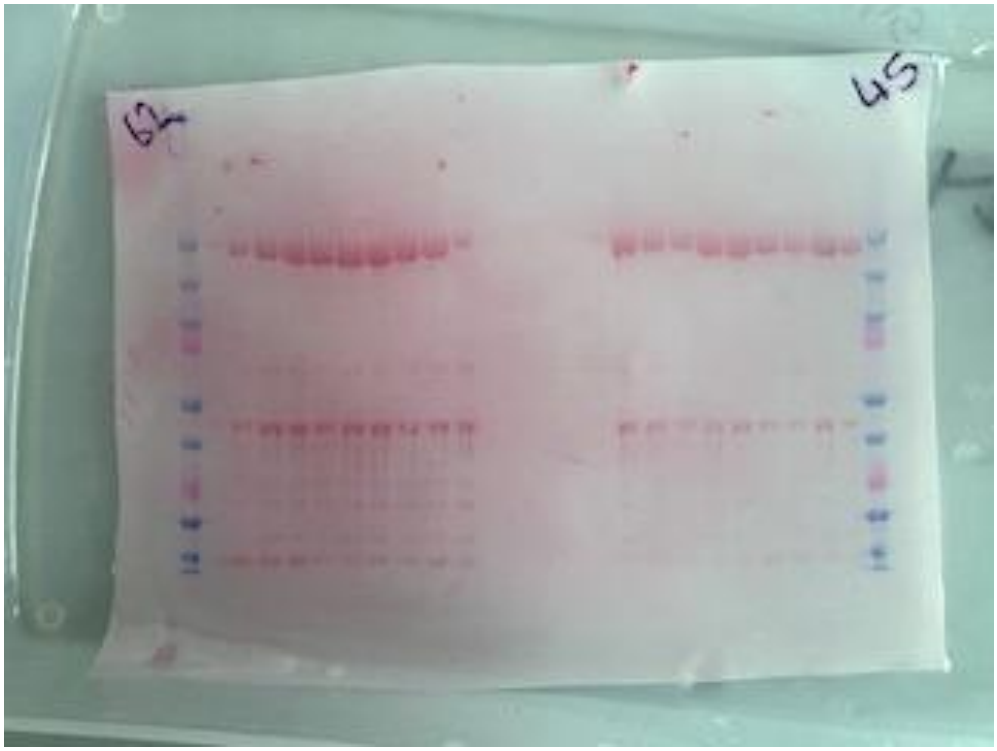


Figure 6- Image of membrane following Ponceau S staining of PVDF membrane

Blocking of membrane

The membrane was then incubated in 3% BSA (Sigma-Aldrich; USA) in TBST (TBS + 0.1% Tween-20) for 1 hour at room temperature allowing the blocking of non-specific binding sites. This step is crucial due to the membrane's high affinity for binding of both protein and antibodies, therefore blocking reduced background noise when detection is completed. Ensure the membrane remains fully immersed throughout the incubation, this was completed through putting

the membrane into a 50ml falcon tube (Corning, USA) ensuring no-overlapping of the membrane.

Primary antibody probing

The membrane was then incubated with the primary antibody overnight at 4^{o}C in a 50ml falcon tube again with gentle rolling at 4*C. The primary antibody was specific to the target protein of interest. Concentrations were dependent on which antibody is being used but typically 1 in 1000. Each falcon tube was loaded with 3-5ml of antibody solution in 3% BSA in 1x TBST (Taylor et al. 2013; Begum et al. 2022).

DAY 3

Washing and secondary Antibody probing

Blots were removed from the cold room and washed for 5x3 min in TBST. The washing step was completed to remove any excess unbound primary antibody. During this stage, the secondary antibody was then diluted in 2.5% BSA in 1x TBST containing horseradish peroxide (HRP)-conjugated secondary antibody in a 1:1000 conc. Following washing, the membrane was placed again in a falcon tube and incubated for 1hr at room temp with gentle agitation (Gingrich et al., 2000). The secondary antibody used was specific to the primary antibody. The primary antibody can be collected and reused through storing in -20°C freezer.

Imaging

Following the incubation, the membranes were then washed with TBST for 5 mins each to remove any unbound antibody. Bio-Rad ECL reagent (Bio-Rad; USA) were then added to the membrane for detection. The membrane was then incubated for 3-5 mins before being placed on the reader (Invitrogen, USA, iBright 1500) for chemiluminescence detection, where the membranes bound HRP luminol is oxidised which emits light. This then shows as bands which can be quantified (Bronstein and Kricka 1989; Nelson and Kacian 1990).

Stripping and Re-probing

In some cases when there is a significant difference in molecular weight of the protein (approximately 30 kilodaltons (kDa), it is possible to strip the membrane of the primary and secondary antibody and “re-probe” in another primary antibody to show a different band, this is a resource and time efficient way to determine multiple proteins from one gel. This is highly useful for the loading control used. This is a tricky step due to the ability to over or under strip the membrane, if left too long the protein can be stripped from the membrane and if not left long enough there will still be the previous primary antibody for the secondary to bind too. PVDF membranes allow the possibility to strip and reprobe as they are more chemically inert and robust (Sennepin et al. 2009; Kar et al. 2012).

Following imaging, the membrane was washed in TBST to remove ECL reagent before the stripping buffer was added, the membrane was then incubated for 15-30 mins before being washed in TBS. The secondary antibody can be added again for 5-10 mins, and the membrane be re-imaged to confirm the stripping of the membrane was successful. Ponceau was added following this step to confirm the membrane was not stripped of the protein entirely (follow steps under confirmation to prep membrane for primary antibody again). The membrane can then be re-blocked for 30 mins in BSA before repeating steps from “primary antibody probing” (Bass et al., 2016).

Coomassie stain

It is well established B actin (alongside Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and tubulin) expression can vary considerably between individuals, disease states, experimental conditions which raises questions to its reliability as a loading control, particularly in settings like inflammation, cancer and metabolic stress where skeletal and metabolic proteins are disregulated (Eaton et al., 2013). Therefore, the use of total protein normalisation approaches such as Coomassie staining has become increasingly recommended as a more robust and consistent strategy to normalise protein abundance as the ability to reflect the total amount of protein

in the lane opposed to the single protein expression. This is also an alternate method for when the protein being probed for is of similar mw to the antibody stained for, e.g. citrate synthase (54mw) is too close to B actin (37mw) to strip and re-probe.

To make the Coomassie Staining solution, 0.1g Coomassie Brilliant Blue R-250 (1%) is diluted in 50ml methanol (50%) and 50ml ddH2O (50%) (Fisher Scientific, USA). It is common practice for some to use 10% acetic acid in this solution; however, it was found that this caused over binding and cause too strong a background which was hard to reverse. The membrane was then incubated in this solution which binds non-specifically to most proteins for 210minutes (dependent on the membrane and how well the Coomassie binds). To then destain the membrane the membrane is washed in ddH_2O until the bands become clearly defined and quantifiable. If the Coomassie has a strong affinity to the membrane to reduce the background noise 50% methanol solution can be added to the membrane to help reduce background noise. The membrane is then left to dry and imaged 24hrs later when the bands are best defined (Welinder and Ekblad, 2011).

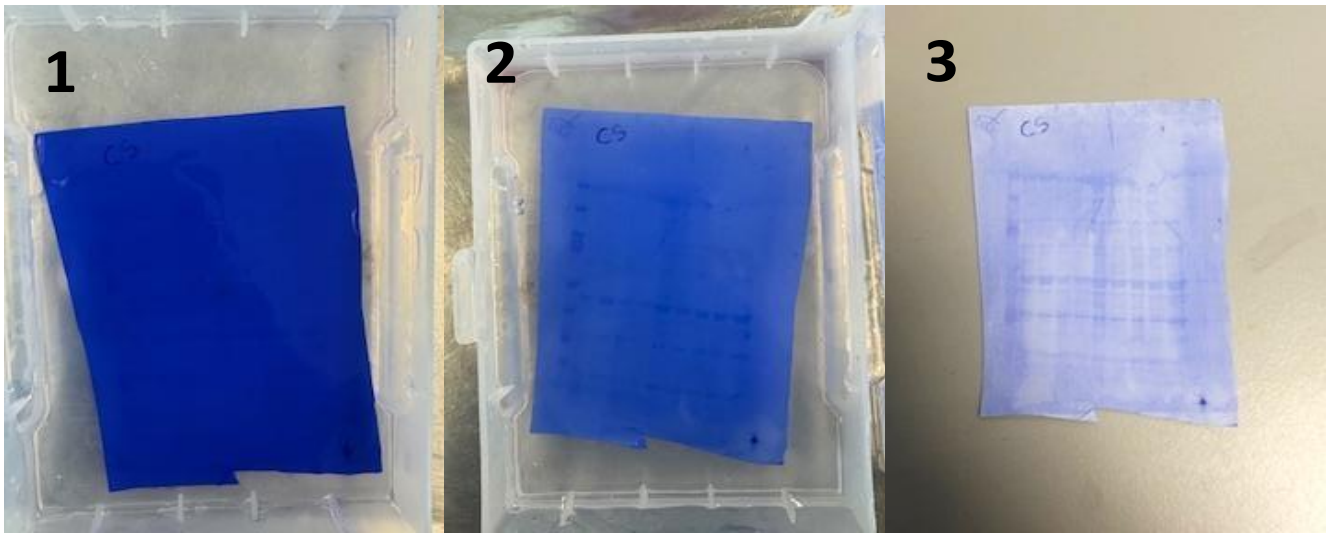


Figure 7- process of destaining a Coomassie membrane to allow for protein quantification. Image shows the membrane at 3 steps: (1) immediately following Coomassie staining, (2) The following destaining using 50% water and 50% Methanol, (3) following morning.

Quantification

Once imaging has been completed for both the antibody probed for and the loading control/ Coomassie stain, quantification of the bands was completed through densitometry in Fiji (Schindelin et al., 2012). The band was selected, and a histogram is drawn highlighting density of the band. This can be completed for all samples before then dividing the antibody probed for by the housekeeping protein to give a normalized abundance (Gassmann et al., 2009).

3.2.9 Bead-Based Multiplex Human Inflammation Panel Protocol

Reagent prep

All reagents were prepared a stated in the LEGENDplex multi-analyte flow assay kit (BioLegend, USA) manual. The pre-mixed beads were sonicated for 1 minute to resuspend, wash buffer was diluted with ddH_2O, and the standard cocktail was reconstituted with 250ul assay buffer.

Standard prep

75uL assay buffer was added to each tube before a 1:4 serial dilution was completed through transferring 25ul of the prior standard. Raw assay buffer was used as the blank to allow a standard curve to be created.

Sample loading and Incubation

37.5uL of assay buffer was loaded into each well with 25uL sample/STDs on top, 12.5uL of the premixed beads was added then added into the well. The V-bottom filter plate was then covered with a plate sealer and incubated on a rocker for 2hrs - shaking at 800 RPM.

Wash

The assay plate was then spun down at 1000rpm for 5mins allowing the beads to pellet together. The supernatant was removed through inverting and flicking the plate in one continuous and forceful motion, and the plate was blotted on blue roll to dry, with care taken to not disturb the pellet. The plate was then washed by pipetting 150uL wash buffer into each well, before then repeating the vortex step. The supernatant was removed again, and the plate was washed again with 200uL with buffer before being span again at 1000 rpm for 5mins, and the supernatant was removed again.

Detection antibody

650uL of detection antibody was mixed with 650uL assay buffer, before 25uL was added to each well. The plate was then sealed and covered with foil before being shaken at 800 RPM for 1hr.

SA-PE step

After 1hr incubation mix 650uL Streptavidin-R-Phycoerythrin (SA-PE) with 650uL assay buffer, and 25uL SA-PE was added to each well before being sealed, covered and shaken for a further 30mins at 800rpm. The plate was then washed and vortexed again as previously stated. The supernatant was removed again, and 150uL of wash buffer was added to each well, with the beads being resuspended by repeatedly pipetting.

Flow cytometry

The flow cytometer (Beckman, USA) was set to plate mode and the bead capture limit set to 4000. Gates were created to capture all beads without collecting debris. The samples were then read and data analysed using BioLEGEND's LEGENDplex data analysis software (BioLegend, USA).

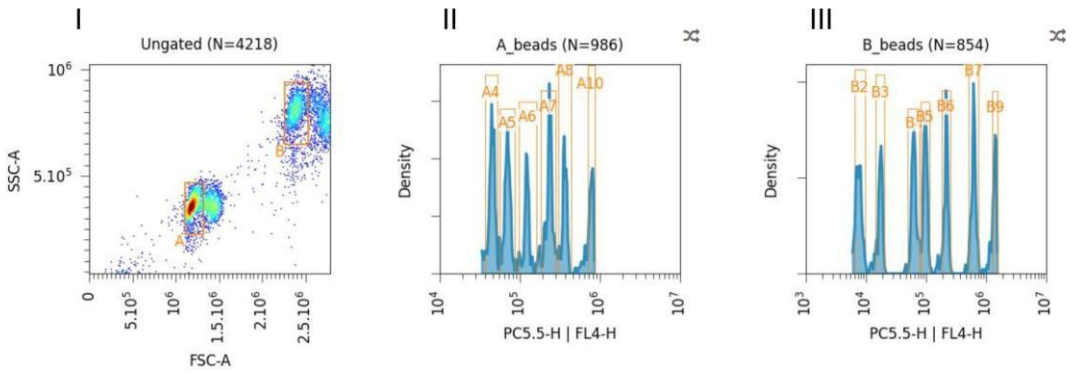


Figure 8- Gating of beads during flow cytometry showing the separation of the 2 bead populations (A and B) with the different beads in those populations. (I) shows the initial gating to remove debris from analysis, (II) shows the 6 different and quantity of beads found in the "A" bead cluster, (III) shows the 7 bead types and the quantity found in the "B" bead cluster. Figure shows a screenshot of the LEGENDplex data analysis software (BioLegend, USA).

3.2.10 Statistical Analysis

Data were first assessed for normality using Shapiro-Wilk tests. Once missing values were imputed using Monte Carlo method (IBM SPSS Statistics (Version

30.0)) (this includes when biopsies were not taken, or housekeeping protein was not present). Note if the housekeeping protein was present, but no blot was present for the protein of interest, then this has been classed as a true 0 and has been included in statistical analysis.

Mixed-model repeated measure ANOVA analysis was used to analyse the changes between the two groups at different time points. Tukey's correction was applied for multiple pairwise comparisons Correlations were run between previously collected Cardiopulmonary exercise testing (CPET) data and baseline and changes to skeletal muscle analytes. A Monte-Carlo imputation was completed for missing data where biopsies were not taken. Data analysis and figure preparation were conducted using GraphPad Prism 10 (GraphPad Software 2365 San Diego, USA). Figures if representative blots were created using GIMP (GIMP 3.1.4, GPLv3). All data is expressed as mean ± standard deviation (SD) unless otherwise stated. Statistical significance was set at $p < 0.05$ as a practical balance between type I and type II errors, where findings are unlikely due to chance.

An exploratory analysis was completed using the predicted concentrations which were classed as outside the limits of detection of the bead-based multiplex assay. Using these predicted values, two-way ANOVAs were completed to assess the differences between treatment groups and changes across the time points of baseline, preoperative and post-operative biopsies. Post-Hoc analysis was completed using Tukey's multiple comparisons to identify pairwise differences between timepoints and groups.

3.3 Results

3.3.1 Demographic Data

Demographics are displayed in table 1.

Table 1- Demographic data of groups showing Average and standard Deviation

Demographic characteristic	SG	PG

Age (years)		66.0 (14.8)	67.5 (8)
Sex	Male	11	11
	Female	6	0
ASA score		2 (1)	2 (1)

ASA: American Society of Anaesthesiologists, SG: Standard care Group, PG: prehabilitation group

3.3.2 Baseline Correlations between $\dot{V}O_2$ peak and Mitochondrial proteins

Baseline muscle biopsy data were analysed to determine the associations between $\dot{V}O_2$ peak and a panel of mitochondrial protein markers. No statistically significant correlations were identified for any of the eight proteins examined (list here, $p > 0.05$). Mitofusin-2 (Mfn2) demonstrated no significant correlation ($r = 0.19$, $p = 0.3481$), alongside DRP-1 which also showed none. ($r = –0.25$, $p = 0.2513$) or Citrate synthase (CS), which also showed no correlation ($r = –0.10$, $p = 0.6447$).

Similarly, no significant relationships were observed for the mitochondrial respiratory chain complexes. There was no significant correlation found between $\dot{V}O_2$ peak and NDUFB8 (Complex I) ($r = 0.25$, $p = 0.2563$), SDHB (Complex II)($r = –0.09$, $p= 0.72$), UQCRC2 (Complex III) ($r = –0.20$, $p = 0.3544$), MTCO1 (Complex IV) ($r = 0.08$, $p = 0.7039$), or ATP5A (Complex V) ($r = –0.17$, $p = 0.4828$). Collectively, these findings indicate that, at baseline, in this specific population, the mitochondrial protein expression levels did not strongly associate with aerobic capacity.

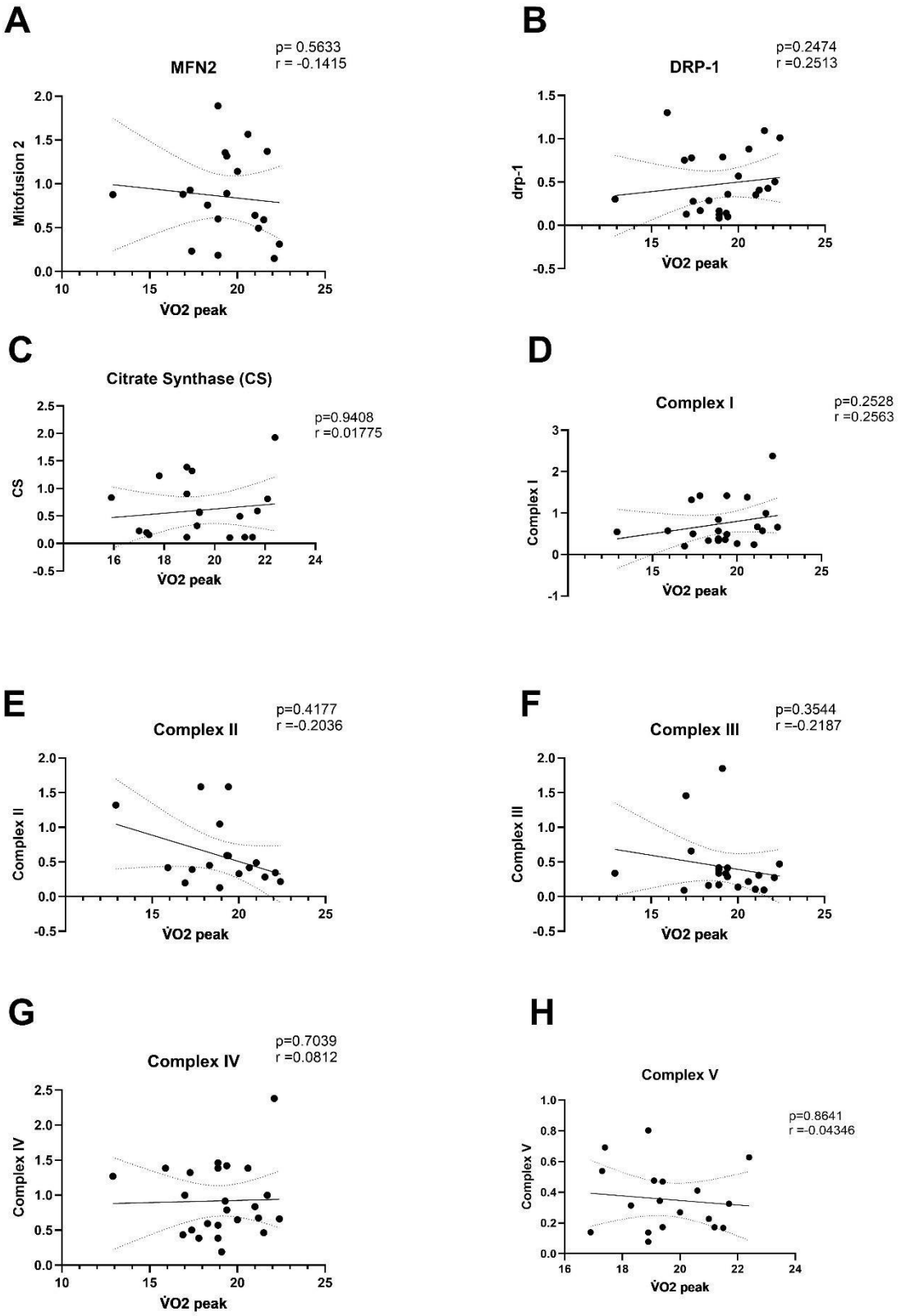


Figure 9- Baseline correlations between mitochondrial protein expressions and $\dot{V}O_2$ peak Relationships are shown for (A) MF2, (B) DRP-1, (C) citrate synthase (CS), (D) NDUFB8 (Complex I), (E) SDHB (Complex II), (F) UQCRC2 (Complex III), (G) MTCO1 (Complex IV), and (H) ATP5A (Complex V). Associations were assessed using Pearson's product–moment correlation coefficient. Standard group: n = 16; Prehabilitation group: n = 7.

3.3.3 Standard Care VS Prehabilitation

Inflammatory Markers

IL-6 demonstrated a significant difference over time ($p < 0.001$, $F(1.7,42.6) = 17.9$), with post-hoc analysis revealing significant decrease in IL-6 from preoperative to post-operative in both standard care ($p = 0.004$) and prehabilitation ($p= 0.027$). No overall group difference or interaction was observed (Prehab: $p = 0.789$ $F(1,25) =0.1$; (Time x Prehab: $p = 0.336$ $F(1.71,42.66) =1.09$).TNFalpha levels remained relatively stable across all timepoints in both groups, with no significant time effect ($p = 0.7872$ $F(1.24, 32.27) = 0.1182$), group effect ($p = 0.1477$ $F(1,26) = 2.277$), or time-group interaction ($p = 0.3292$ $F(1.24, 32.27) =1.052$).

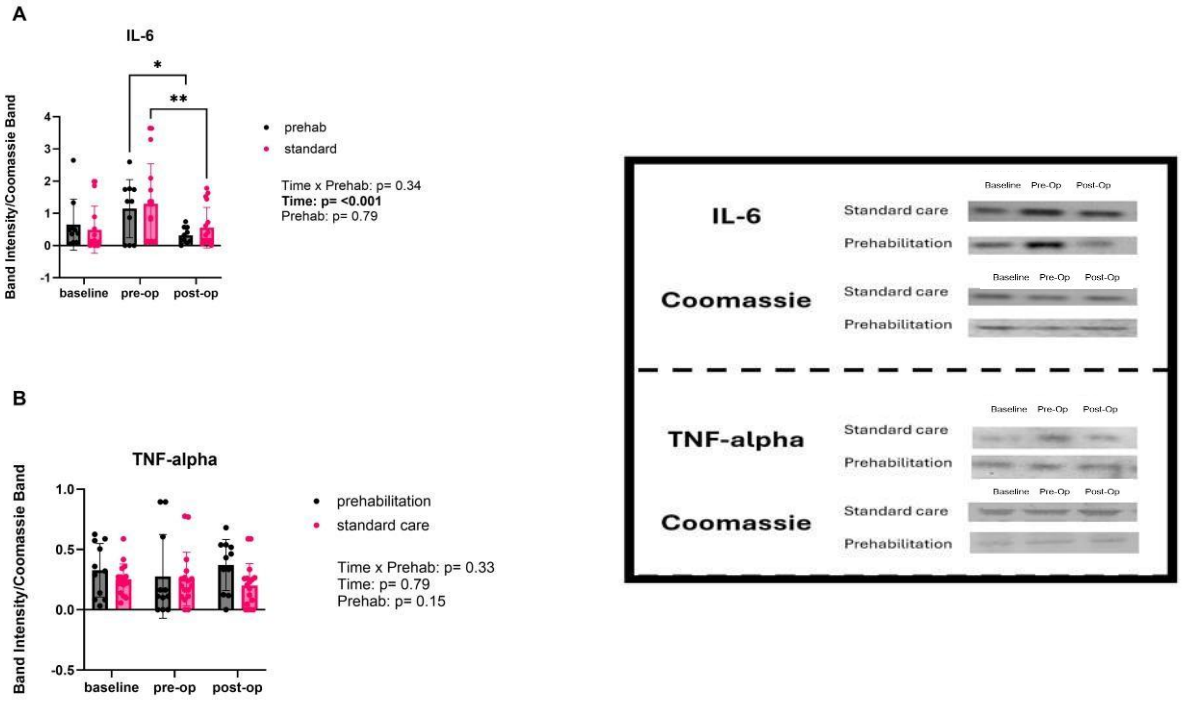


Figure 10. Inflammatory marker expression following HIIT prehabilitation versus standard care. *(A) Interleukin-6 (IL-6) and (B) tumour necrosis factor-alpha (TNF-alpha). Data is presented as band intensity normalized to Coomassie loading control (mean ± SEM). *p < 0.05, **p < 0.01 between groups at individual timepoints. Statistical results for main effects and interactions are displayed in each panel.* Representative blots are showcased.

Anabolic Signalling Markers

AMPK expression showed no changes through the surgical timeline (p = 0.2049, F (1.86,46.55) =1.648) or between groups (p = 0.147, F (1,25) =2.2). Nor was there any interaction (p = 0.731, F (1.86,46.55) =0.2). FoxO3a demonstrated significant differences over time (p = 0.002, F (1.57,41.00) =8.7) with a significant increase from pre-operative to post-operative levels in both groups (prehab: p= 0.03, standard care p=0.03). The standard care group showed a significantly higher FoxO3a expression at post-operative biopsy when comparing to baseline (p = 0.03) however, this significance was not present in the standard care group).The prehabilitation group showed higher mean

FoxO3a expression at all time points, though no overall group effect was detected (Time x Prehab: $p = 0.231$ $F(1.57,41.00) = 1.521$, Prehab: $p = 0.172$ $F(1,26) = 1.97$).

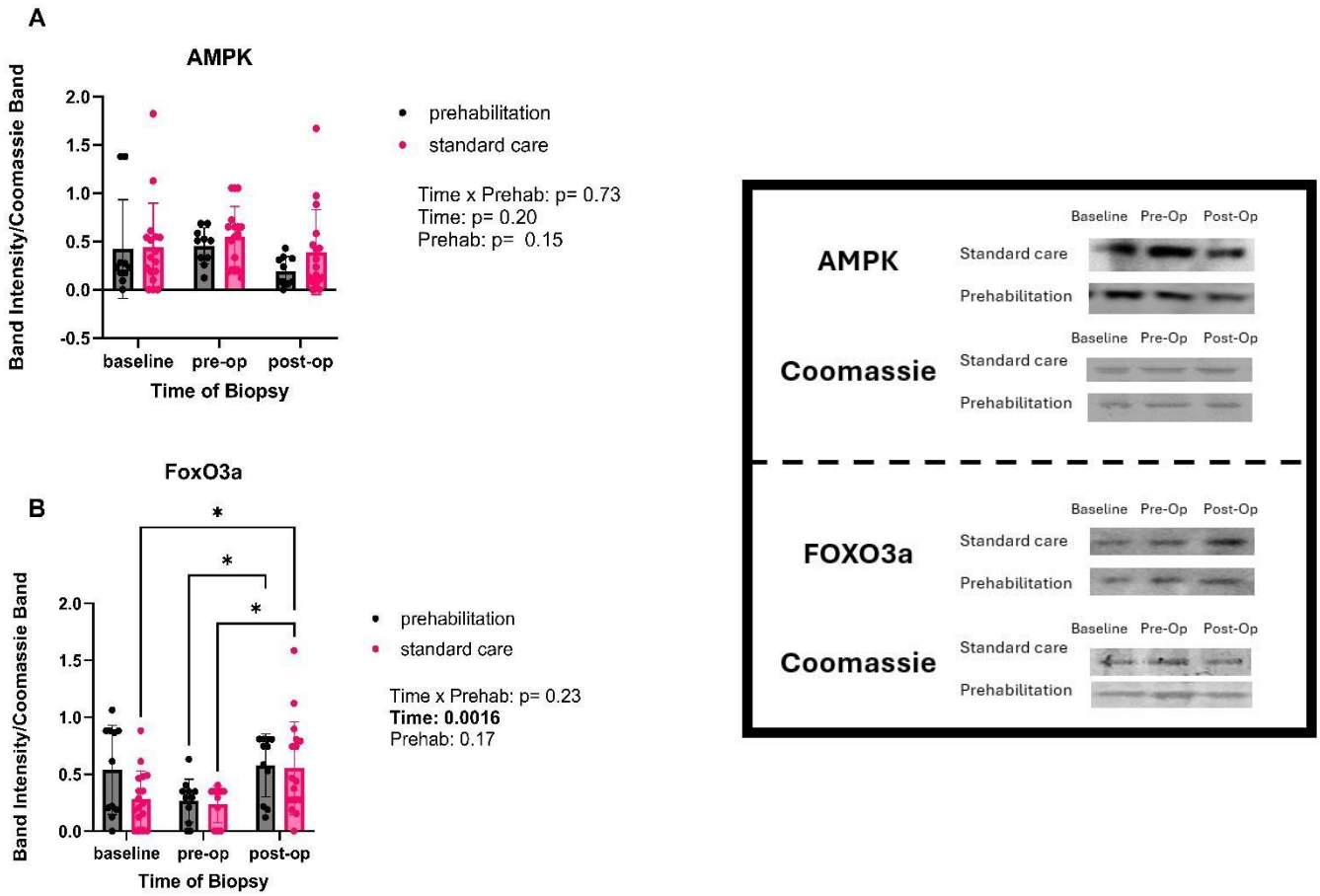


Figure 11. Anabolic signalling pathway expression following HIIT prehabilitation versus standard care. *(A) AMP-activated protein kinase (AMPK) and (B) Forkhead box protein O3a (FoxO3a) protein expression levels measured at baseline, pre-operative, and postoperative timepoints. Data is presented as band intensity normalized to Coomassie loading control (mean ± SEM). Black circles represent prehabilitation group, pink circles represent standard care group. Statistical results for main effects and interactions are displayed in each panel. Representative blots are showcased*

Mitochondrial Electron Transport Complexes

Mitochondrial electron transport complexes showed variable responses to prehabilitation. Complex I (NDUFB8) exhibited a statistically significant difference in groups (p=0.0004, F (1,26) =16.57). There was no significance found for time (p=0.55, F (1.52,39.62) =0.51) or interaction (p=0.08, F

(1.52,39.62) = 2.8). Complex II demonstrated a significant main group effect (p=0.01 F (1,26) =6.8) but no effect of time (p=0.35, F (1.58,41.30) =1.0). There was, however, a significant interaction (p=0.01, F (1.6,41.30) =6.4). Post-hoc analysis found a significant decrease between pre and post operative levels in the standard care group (p= 0.04). Complex III showed no changes in prehab effect (p= 0.2588 F (1,26)1.3), or time effect (p= 0.4332 F (1.61,41.90) 0.8). However, there was a significant interaction (p= 0.004 F (1.61,41.90) 7.2). Post hoc analysis highlighted a significant decrease between preoperative and postoperative biopsies in the standard care group (p=0.05). Complex IV showed no difference in time (p= 0.15, F (1.68,43.68) 2.0) or group (p= 0.49, F (1,26) 0.5). However, there was a significant interaction (p= 0.0018, F (1.68,43.68) 8.1). Post-hoc analysis highlighted a significant decrease between preoperative and postoperative biopsies in the standard care group (p=-.034). This decline in Complex IV expression in the standard care group was not observed in the prehabilitation group (p = 0.17) Complex V showed no time effect (p=0.3735, F (1.874,48.73) 0.9925), no difference in group (p=0.2609, F (1,26) =1.321), and no significant interaction (p=0.22 F (1.874,48.73) 1.568).

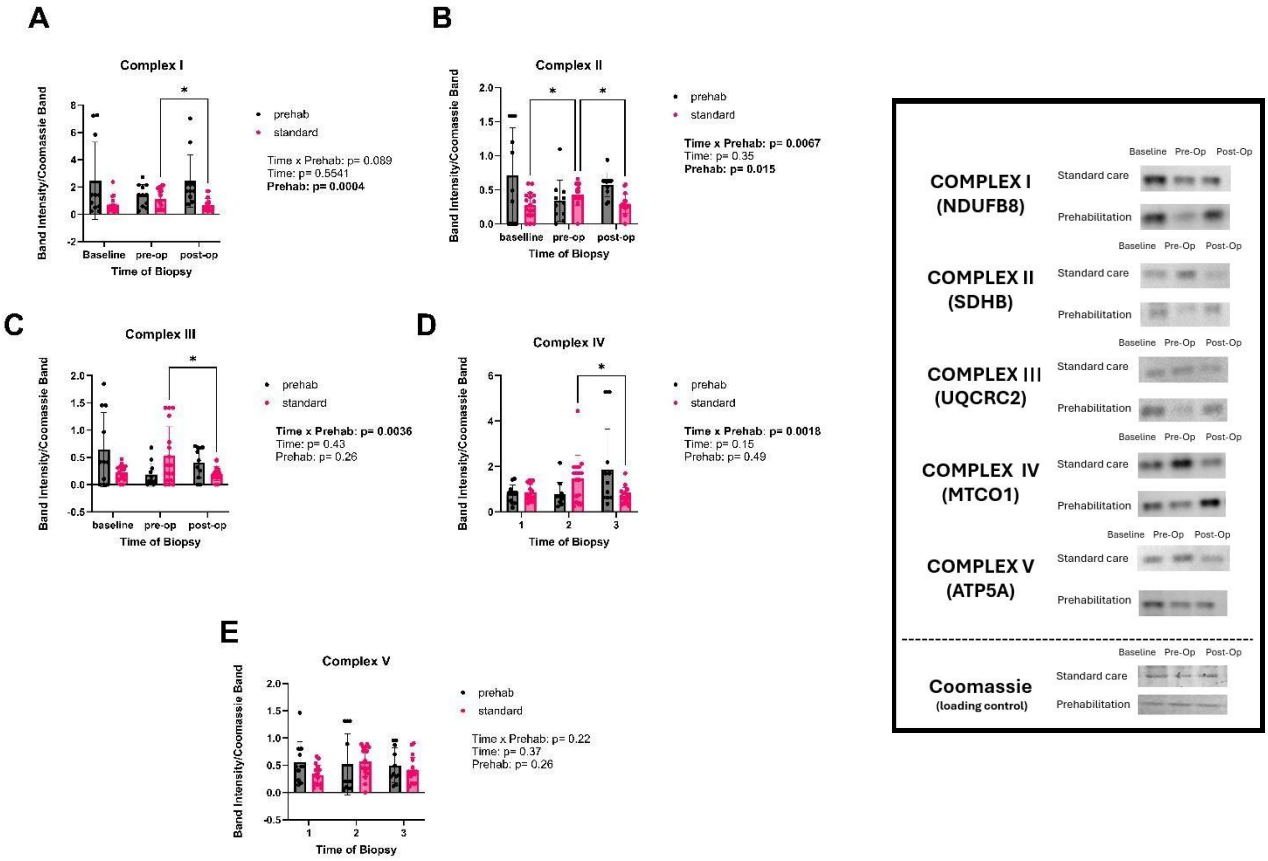


Figure 12. Mitochondrial electron transport chain complex expression following HIIT prehabilitation versus standard care. *Protein expression levels of (A) Complex I, (B) Complex II, (C) Complex III, (D) Complex IV, and (E) Complex V measured at baseline, pre-operative, and post-operative timepoints. Data is presented as band intensity normalized to Coomassie loading control (mean ± SEM). *p < 0.05, **p < 0.01 between groups at individual timepoints. Statistical results for main effects and interactions are displayed in each panel. Representative blots are shown on the right-hand side.*

Mitochondrial Protein Markers

Mitochondrial protein markers revealed Mitofusion-2 showed no time effect (p=0.08, F (1.28,33.49) = 3.1), no difference in group (p=0.32, F (1,26) =1.0) and no significant interaction (p=0.08, F (1.28,33.49) = 1.2). DRP1 demonstrated a significant difference in time effect (p= 0.04, F (1.70,44.26) = 0.4), with post-hoc

analysis revealing significant increase in DRP-1 from preoperative to post-operative in standard care (p=0.02). No overall group difference or interaction was observed (interaction: p = 0.85 F (1.7,44.26) = 0.1, group: p = 0.28 F (1,26) =1.2). Citrate synthase showed no time effect (p=0.1608 F (1.7,44.78) = 1.9), no difference in group (p=0.99 F (1,26) =1.01) and no significant interaction (p=0.53, F (1.72,44.78) = 0.01).

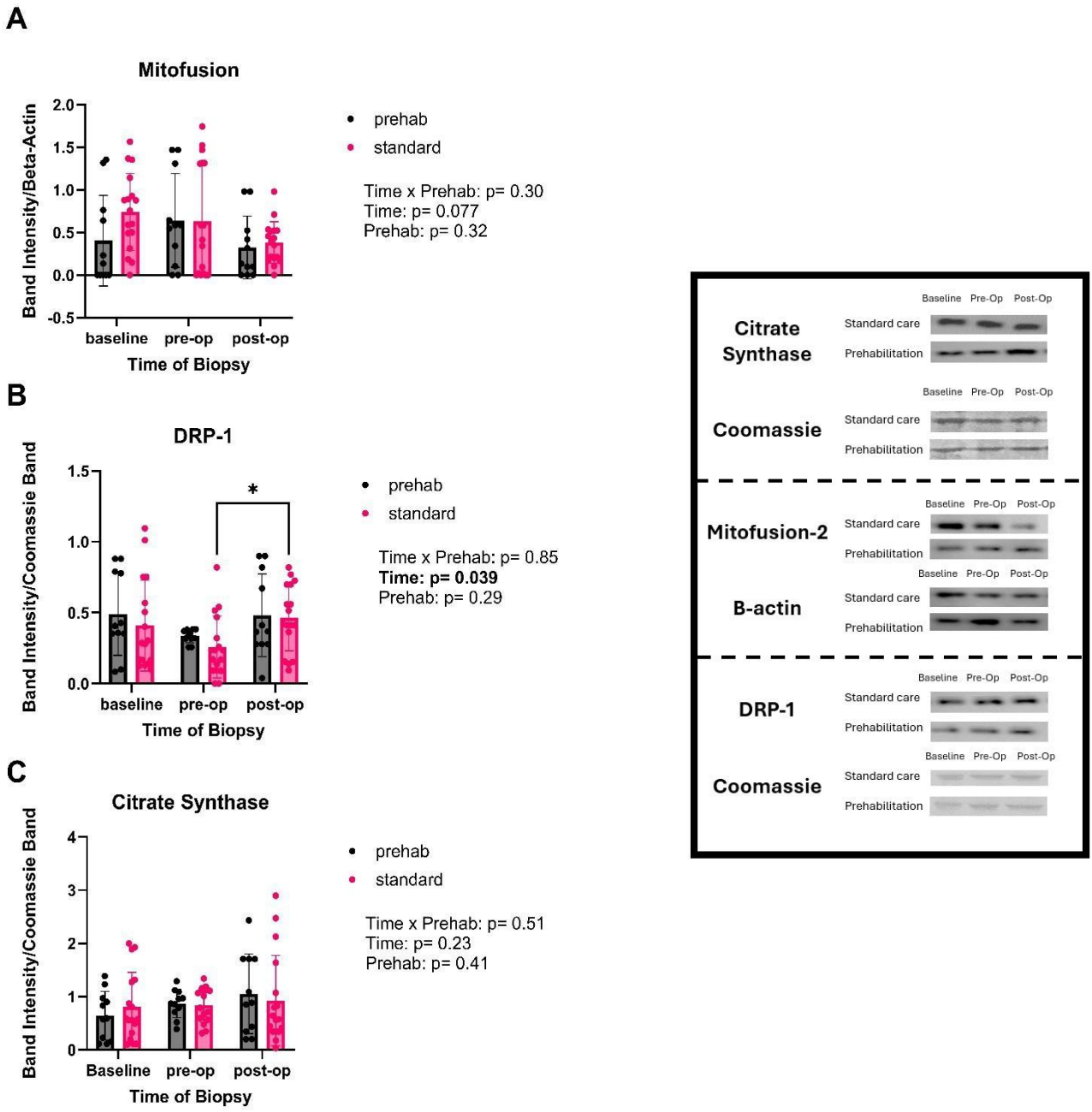


Figure 13. Mitochondrial dynamics and biogenesis marker expression following HIIT prehabilitation versus standard care. *Protein expression levels of (A) Mitofusin-2, (B) dynamin-related protein 1 (DRP-1), and (C) citrate synthase measured at baseline, pre-operative, and post-operative timepoints. Data are presented as band intensity normalised to Coomassie loading control (mean ± SEM). Statistical results for main effects and interactions are displayed in each panel. Representative blots are shown on the right-hand side.*

Multiplex Inflammation Panel Assay

Table 2- results of LEGENDplex bead based multiplex assay for inflammatory cytokines.

	Baseline Biopsy (25 samples)		Preoperative Biopsy (14 samples)		Post-operative Biopsy (23)	
Cytokine	Detected Number	Concentration (pg/ml)	Detected Number	Concentration (pg/ml)	Detected Number	Concentration (pg/ml)
IL-1β	3	(30.3±22.4)	4	(62.6±27.0)	4	(94.5±76.5)
IFN-α2	6	(64.0±57.8)	0	ND	3	(157.5±29.1)
IFN-γ	0	ND	0	ND	0	ND
TNF-α (TNFSF2)	0	ND	0	ND	0	ND
CCL2 (MCP-1)	0	ND	0	ND	0	ND
IL-6	0	ND	0	ND	0	ND
CXCL8 (IL-8)	9	(16.6±9.42)	5	(11.7±4.2)	9	(14.3±9.4)
IL-10	0	ND	0	ND	0	ND
IL-12p70	0	ND	0	ND	0	ND
IL-17A	0	ND	0	ND	0	ND
IL-18	11	(81.7±80.6)	10	(50.0±25.7)	14	(87.6±58.0)
IL-23	0	ND	0	ND	0	ND
IL-33	0	ND	0	ND	0	ND

Following analysis of the tissue using the Multiplex assay, only IL-1β, IFN-a2, CXCL8 (IL-8) and IL-18 gave were detectable in samples (see table 1).

Time Effects

Surgery induced significant changes in circulating inflammatory cytokine levels (Table 1). Significant time effects were observed in IL-1β ($p = 0.0035$), IFN-γ ($p = 0.0279$), TNF-α ($p = 0.0181$), IL-12 ($p = 0.0154$), IL-17 ($p = 0.0341$), IL-18 ($p = 0.0358$), and IL-23 ($p = 0.0041$). Post-hoc pairwise comparisons only revealed specific between-group differences at individual time points for IL-18, IL-17, and IL-23: prehabilitation group showed significantly difference in concentrations between baseline and post operative biopsies in IL-18 and IL-23 ($p= 0.0494$ and 0.0451 respectively); whereas IL-17 post hoc analysis highlighted a significant difference between pre-operative and post-operative concentrations in the standard care group ($p=0.03$).

Prehabilitation Effects

Five cytokines showed significant differences between the prehab and the standard care group in a range of directions (see figure 14): IFN-γ ($p = 0.0171$, $F (1,26)$ 6.6), CCL2 ($p = 0.0476$, $F (1,16)$
4.6), IL-8 ($p = 0.0121$, $F (1,26)$ 7.3),), IL-10 ($p = 0.0110$, $F (1,26)$ 7.5), and IL-18 ($p = 0.0013$, $F (1,26)$ 7.5).

Interaction Effects

There were no significant interactions observed in any of the 13 cytokines ($p > 0.05$).

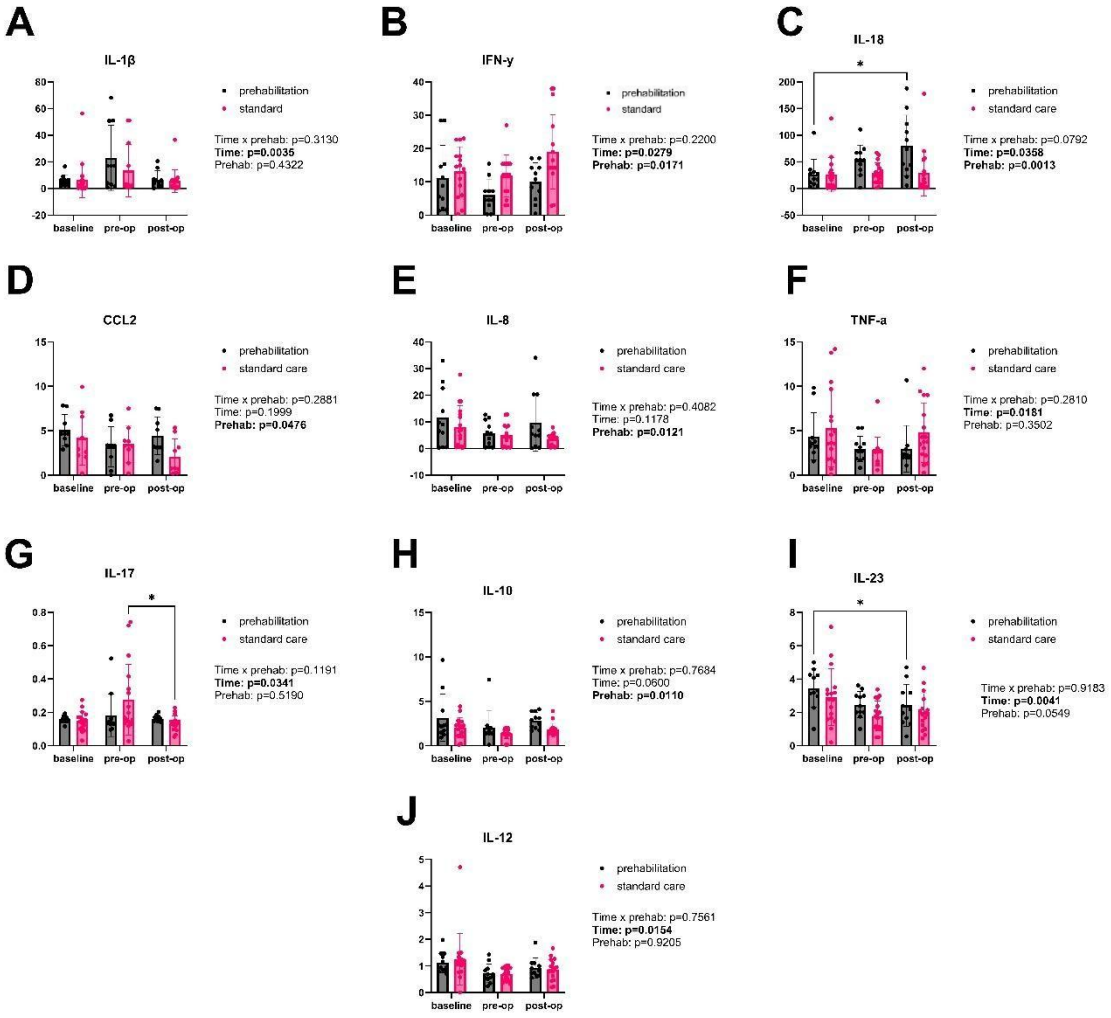


Figure 14. Inflammatory Cytokine Profile Assessed by LEGENDplex Multiplex Assay Plasma concentrations of key inflammatory markers. Bars represent mean ± SEM. Statistical significance was assessed using repeated measures ANOVA with post hoc corrections. $p < 0.05$, $\mathbf{p} < 0.01$, $\mathbf{p} < 0.001$ indicate significant differences across timepoints or between groups. Only cytokines which presented significant results are shown, IFN-a2, IL-6 and IL-33 were all also analysed however not shown in results.

Correlations Between Mitochondrial Electron Transport Chain Markers and changes in anaerobic threshold Changes Following Home Based Prehabilitation

Prior findings have shown a significant improvement in anaerobic threshold following prehabilitation. Therefore, correlations were undertaken to examine the relationship between changes in mitochondrial protein expression and anaerobic threshold improvements following prehabilitation before surgery.

Mitochondrial Dynamics and Anaerobic Threshold

No significant correlations were found for mitochondrial dynamic proteins: Mitofusin ($r = 0.38$, $P = 0.31$) or DRP-1 ($r = -0.43$, $P = 0.22$).

Mitochondrial Content and Functional Capacity

Citrate synthase showed no correlation ($r = -0.06$, $P = 0.89$).

Electron Transport Chain Complexes and Functional Capacity

No significant correlations were found between AT and Complex I (NADH dehydrogenase) ($r = -0.06$, $P = 0.80$), Complex II (succinate dehydrogenase) ($r = 0.52$, $P = 0.13$), Complex III (cytochrome bc1 complex) ($r = 0.08$, $P = 0.84$), Complex IV (cytochrome c oxidase) ($r = 0.01$, $P = 0.87$), or Complex V (ATP synthase) ($r = -0.10$, $P = 0.79$).

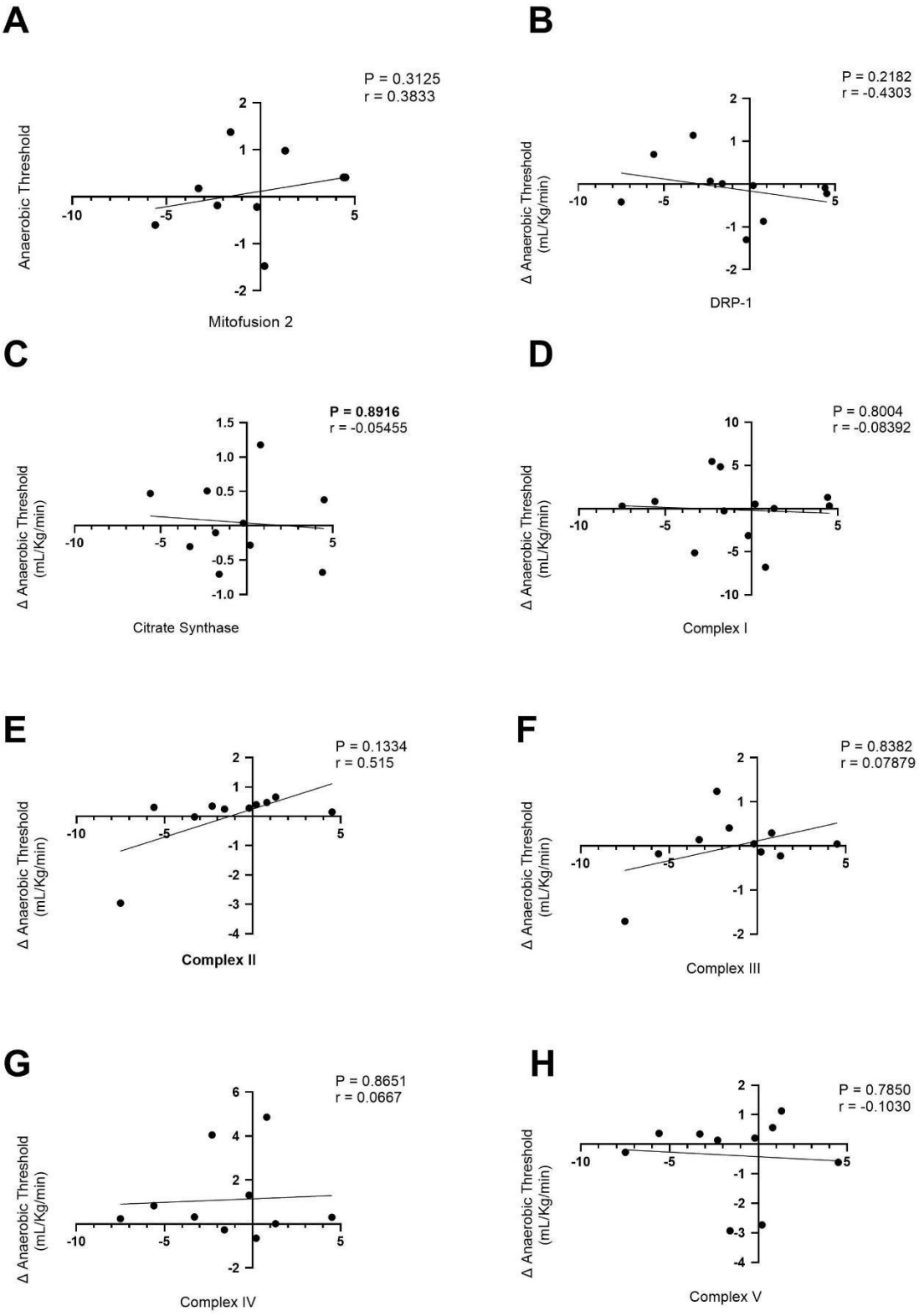


Figure 15- Correlations between changes in mitochondrial markers and changes in anaerobic threshold following HIIT prehabilitation. Scatter plots show the relationship between the changes in mitochondrial markers and Anaerobic threshold.

3.4 Discussion

Principal Findings

The study presents novel observations into skeletal muscle adaptations relating to prehabilitation, and the impact of HPB and CR cancer surgery. A home-based prehabilitation programme provides molecular adaptations that may contribute to improved surgical outcomes. In similar cohorts, home based prehabilitation was shown to improve anaerobic threshold in the 2–4-week period (Blackwell, 2023). Therefore, this study mainly aimed to identifying protein markers in skeletal muscle which may offer multi-scale modelling to connect the molecular trigger to the physiological response.

The most significant finding of this study was the protective effect found in the mitochondrial electron transport chain complexes. In the standard care group, significant decreases were observed in complex I-IV from preoperative to postoperative timepoints suggesting widescale mitochondrial disfunction following surgery. However, the prehabilitation group seemed to have maintained similar complex I-IV expression over the same period, indicating that prehabilitation prior to surgery may have a protective effect against the mitochondrial damage induced in surgery.

Electron Transport Chain Preservation

The preservation of mitochondrial respiratory complexes I-IV potentially has exciting clinical implications. Complexes I (NADUFB8) and II (SDHD) are responsible for the transfer of electrons from NADH and succinate respectively; therefore, the impaired expression seen in the standard care group suggests impaired cellular energy transfer following surgery (Cecchini 2003; Bandara et al. 2021). Similar findings in Complex III (UQCRC2) and IV (MTCRO1) further support this finding of impaired cellular energy transfer, Complex III transfers electrons from ubiquinol to cytochrome C (Cyt-C) before Complex IV then uses electrons from Cyt-C to reduce oxygen to water (Guo et al., 2013). The finding that prehabilitation may prevent this decline is particularly of interest in Complex IV. Studies in mice have found complex IV disfunction to correlate to increased

reactive oxygen species (ROS) and a decrease in lifespan. In a clinical setting this increase in ROS alongside mitochondrial disfunction may cause impaired recovery and a reduction in functional capacity therefore lengthening stays (Reichart et al., 2019).

Mitochondrial Dynamics and Cellular Health

Interestingly, unlike in the mitochondrial complexes, DRP-1 expression in the standard group saw an increase from preoperative to postoperative ($p=0.0156$) biopsies. This finding therefore indicates enhanced mitochondrial fission, which is typically associated with cellular stress responses and mitochondrial quality control mechanisms (W. Li et al., 2025). This increase was not found in the prehabilitation group suggesting increased physiological stress was faced by the standard care group requiring greater mitochondrial fission as a protective function to remove damaged mitochondria, unlike the prehabilitation group which saw a more balanced surgical response (Zerihun et al., 2023). Interestingly, we observed no significant changes in Mitofusin-2 (MF2) expression, which is a marker of mitochondrial fusion. This finding therefore suggests mitofusion-2 remained stable throughout the surgical timeline, this may be because the timeline of biopsies missed when fusion was expressed higher (potentially further down the recovery timeline opposed to acute stress response) or mitochondrial fusion was supported by changes in activity to Mitofusion-1 or OPA-1 (Song et al. 2009; Joaquim et al. 2025). Future studies could provide further insight through an increase in mitochondrial proteins studied and increased biopsy timepoints post-surgery.

Mitochondrial changes and Activity Tracker Results

Analysis conducted as part of the SPECS trial reported that surprisingly, there was no significant difference in the total activity levels or level of vigorous intensity of the prehabilitation group in comparison to the standard care group ($p>0.05$). One explanation for this may be that despite completing the exercise sessions, the prehabilitation group may be more sedentary outside of the dedicated prehabilitation sessions or that the standard care group increased their activity levels. As a result, this may be why we saw no effect of prehabilitation compared to standard care during the baseline to pre-operative

biopsies. However, the prehabilitation prescribed may have allowed metabolic adaptation to the heightened stress of surgery, hence the protective effect seen (Waller et al., 2021; Whittle et al., 2025).

Inflammatory Response Modulation

Systemic Inflammatory Markers

The significant decrease in IL-6 levels from preoperative to postoperative in both groups (standard care p=0.0041, prehabilitation p=0.0272) highlights a significant improvement in recovery of inflammation from surgery as well as the removal of the tumour itself. Tumours are a key source of IL-6 production which contributes to the inflammatory environments needed to support tumour growth. Surgery therefore removes this source of inflammation (Fisher et al. 2014; Soler et al. 2023). Despite the expectation that IL-6 may increase immediately following surgery it should be noticed the timeframe of postoperative biopsies was 6-16 weeks which has shown to be sufficient time to see the decline in systematic inflammation and tumour-related inflammation to be reversed (Taylor et al. 2023).

Analysis using the multi-bead assay shed further light on the inflammatory responses following surgery. The prehabilitation group showed significantly different concentrations of multiple cytokines across the surgical period, Including IFN-y, IL-8, IL-18 and IL-10. The elevated IL-10 levels in the prehabilitation group are particularly of interest due to the role interleukin-10 plays as an anti-inflammatory molecule. IL-10 mitigates the production of IL-6, TNF-alpha and other inflammatory cytokines causing systematic inflammation (Carlini et al. 2023). However, the assay analysis must be taken with caution as cytokine values were read as below the level of detection for the most part (see table 1). Therefore, this exploratory analysis was completed using the software's predicted values which are classed as below the limit of detection and therefore more exploratory of potential findings for future research.

The results of this across surgical timepoints contributes to the understanding of the benefits of prehabilitation. The possible enhancement in anti-inflammatory IL-10 could reduce the risk of peri-operative complications, lengthened recovery

and comorbidities through mitigating the effect of inflammatory cytokines. As mentioned prior, future research may aim to take additional biopsies in the perioperative period to understand the immediate inflammatory response following surgery for example 24 hours following, 1 week after surgery to better capture the early inflammation response, rather than a biopsy which may show long term recovery (Ge et al., 2025).

Anabolic Signalling and Metabolic Adaptation

FoxO3a Upregulation

The results of the anabolic signalling proteins observed highlighted a significantly higher expression of FoxO3a across the surgical timeline. Post-hoc analysis highlighted changes in both care groups between pre- and post-surgery biopsies. FoxO3a is a transcription factor from the forkhead class ‘O’ family, key regulators of skeletal muscle protein turnover, particularly regarding catabolism. When under stressful conditions, such as metabolic stress or oxidative stress, FoxO translocate to the nucleus to promote the expression of muscle atrophy genes whilst also triggers autophagy, and mitophagy which causes further catabolism in the cell. (Zhou et al. 2012, Sanchez, 2014). The increased expression supports research suggesting that FoxO expression reflects ongoing stress and remodelling. Surgery is notoriously a severe physiological stressor therefore the heightened expression of FoxO, as shown, is expected due to the heightened oxidative stress experienced. The impact of an increase in FoxO3a following surgical stress signals creates a catabolic environment with autophagy induction and highlights a fundamental cellular response (Maiese et al. 2008; Zhang et al. 2013; Webb and Brunet 2014).

Comparing baseline to post-operative biopsies, the standard care group saw an increase in FoxO3a which in the prehabilitation cohort remained statistically insignificant; one reason for this may be the prehabilitation group enhanced metabolic flexibility (Tetlow and Whittle, 2025). Alternatively, the reason for the lack of statistical significance may simply be because baseline FoxO3a concentrations were higher in the prehabilitation group.

AMPK Signalling

Despite the changes seen across the surgical timeline in FoxO3a, surprisingly there was a lack of significant changes in AMPK expression across timepoints and groups. This may be because of flaws in the study design discussed in the study limitations section. The lack of significance found in this study is unexpected due to the central role AMPK plays in metabolic regulation (Dasgupta and Chhipa 2016; Jeon 2016). AMPK promotes mitochondrial biogenesis and enhances oxidative metabolism to generate ATP whilst simultaneously inhibiting protein synthesis to limit energy consuming pathways (Trefts and Shaw, 2021), previous research has highlighted exercise to have mixed effects on AMPK activation, with a study in moderate exercise intensity (like the protocol prescribed here) was shown to have no effect on VO_2 in endurance trained men (McConell et al., 2020).

Correlation with Functional Capacity

Baseline Mitochondrial- $\dot{V}O_2$ Relationships

The lack of significant baseline correlations between baseline mitochondrial proteins and $\dot{V}O_2$ peak previously studied highlights the intricacy of functional capacity and the multiple mechanisms associated with aerobic fitness. Despite contrasting with previous research in healthy populations, the lack of significant correlations may reflect the severity of HPB and CR cancer surgery populations and the pathophysiology in this cohort, alongside the potential for severe mitochondrial disfunction from systematic inflammation and concurrent medical treatments (Cole et al. 2018; Wu et al. 2024). The weak, insignificant correlations observed (ranging from r = -0.25 to r = 0.25) suggest that in this population, cardiovascular and pulmonary factors could be factors effecting aerobic capacity rather than mitochondrial content. This finding highlights the complexity of exercise capacity in all populations and suggests that prehabilitation benefits to functional capacity could occur through multiple mechanisms beyond simple mitochondrial adaptation (Franklin et al. 2022; Maroto-Izquierdo et al. 2025).

Post-Intervention Correlations

Correlations were completed between changes to mitochondrial adaptations and anaerobic threshold at baseline and pre-surgery. Similarly to the baseline correlation, the lack of significance found was unexpected due to the known mechanisms of mitochondria and anaerobic threshold. The moderate positive correlation observed for Complex II ($r = 0.515$, $p = 0.1334$), while not statistically significant, suggests a potential relationship that may become apparent with larger sample sizes. As alluded to earlier, the functional improvements seen in the population may be a result of central cardiovascular adaptations, neuromuscular coordination improvements, or other factors not captured in our mitochondrial analysis (Hughes et al. 2018; Guzzoni et al. 2025). Importantly, changes seen to AT ranged from 50% decline to 50% increase suggesting there was substantial variability in response to the prehabilitation intervention which could obscure potential correlations (Li et al., 2019).

Implications for Clinical Practice

The most significant finding in this study was the molecular evidence of prehabilitation's potential to preserve mitochondrial complexes and influence inflammatory cytokine profile through cancer surgery.

The mitochondrial preservation may contribute to faster recovery and tolerance to surgical stress, giving healthcare providers evidence to encourage patient adherence to prehabilitation programmes. It is well established that the psychological factor of having a sense of control is a key determinate behind prehabilitation uptake, therefore, providing further rationale behind the biological mechanisms to complete prescribed exercise may further improve reasoning for this (van der Velde et al. 2023).

As shown in this study, mitochondrial benefits can be achieved in the relatively short timeframe of 2-4 weeks prior to surgery making prehabilitation a feasible output for all. Furthermore, the remote supervision model demonstrated in this study also supports scalable implementation where resources may be scarce.

Study Strengths and Limitations

Strengths

This study presents novel data exploring the impact of surgery on muscle markers, whilst exploring the effects of a prehabilitation protocol across a surgical timeline in a cancer population. The findings of this study present several molecular advances to aid understanding of the benefits of prehabilitation. The use of muscle biopsies to assess molecular mechanisms is a major strength of this study, as it provides direct evidence of novel tissue level adaptations and addresses a novel gap in the literature. The comprehensive protein analysis using both western blotting and multiplex assays allows for detailed characterisation of cellular responses. A major strength of the study was a randomised-controlled design which allowed for the minimisation of selection bias. Tight inclusion criteria were employed to maximise ecological validity within which prehabilitation must be evaluated.

Limitations

Despite the significant findings of this study there is several limitations which must be noted. One of the key recognisable issues of this study is the sample size, particularly in the prehabilitation group, was the missing data points/missing biopsies. 28 participants gave muscle biopsies (17 standard care and 11 prehabilitation. This was further worsened by the lack of pre-surgical biopsies taken with 13 pre-surgical biopsies missing. Missing data may have occurred due to emergency surgery conditions that limited the available time frame for biopsy collection, and samples which have been collected have been collected had excessive connective tissue and fatty material, effectively diluting the muscle biopsies concentration, and rendering the concentration below the limit of detection.

Despite Monte-Carlo imputations being completed to estimate missing values for the ANOVA analysis, % change correlations cannot be used with imputed data meaning there was a large reduction in the analysable population. This therefore limited correlations between the changes in CPET and mitochondrial enzymes with only 11 participants (4 prehab and 7 standard care) having both the CPET analysis and biopsy taken pre-surgery, as a result potentially underestimating the mitochondrial adaptations.

Furthermore, the intervention may have inadvertently attracted a subset of patients who were fitter, more motivated and more likely to complete the exercise component. Another unavoidable recruitment bias was the effect of the Covid-19 pandemic. Restrictions due to the virus had a dual impact on recruitment. First, local operative guidelines suggested that frail patients who were most at risk of peri-operative complications be managed with alternative treatments such as chemotherapy and other non-operative interventions. Second was the logistical considerations of patients having to attend the hospital site for assessment visits and the risk that posed to contracting Covid-19 with the consequence of either their surgery being delayed or cancelled altogether. Even with risk reduction measures there was a balance needed between ethical and legal considerations of patients participating in the trial while maintaining national restrictions such a quarantine prior to surgery. Alongside the study design issues, the variance in timeframe (two to four weeks), and demographic differences such as age, sex, BMI and concurrent treatments may have influenced physiological responses and contributed to the variation in results seen.

As alluded to earlier, the results of the activity trackers did not differ between the two care groups. Despite this, prior findings suggest the intervention was intense enough to see change cardiovascular change in the short prehabilitation period, however this study highlights the prehabilitation to be insignificant enough to alter muscle protein levels within the timeframe, highlighting the complexity of physiological systems interactions. Alongside activity trackers, food diaries were taken to assess the nutritional intake of the study cohort, however, this parameter was abandoned due to poor adherence. It is well established that nutrition is a key factor in prehabilitation studies, therefore the lack of data collected calls for further investigation into the effects of nutrition in this cohort (West et al. 2017).

Known limitations of western blotting should also be acknowledged, despite careful standardisation (CV less than 5%), there are multiple methodological steps including loading, antibody specificity and detection conditions which

should be acknowledged. Variation in these factors can affect band intensity and therefore cause variability (Bass et al., 2016).

One of the key limitations of the bead-based multiplex assay was working with most data under the limits of detection, this may have been for several reasons including assay sensitivity or low abundance of analytes in the sample. Future studies should endeavour to use higher sensitivity methods for example single molecule counting, radioimmunoassay and mass spectrometry (Dowall et al., 2019).

Future research in prehabilitation should continue to focus on mechanistic changes which are seen through the surgical timeline. Alongside the markers analysed in this population further evidence can be collated by investigating other mitochondrial biogenesis markers, and metabolic flexibility. Furthermore, understanding how muscle-derived factors influence whole-body inflammation could inform optimization of prehabilitation protocols and therefore reduce the risk of perioperative complications, and reduce the rates of readmissions and therefore alleviate the burden on the NHS.

By future research continuing to focus on mechanistic insights, it may allow pharmaceutical interventions to be established. Future studies should also aim to take further biopsies closer to the post-operative period as mentioned earlier, as well as potentially during surgery. Furthermore, different types of prehabilitation protocols should be explored to allow personalised optimisation of protocols and therefore optimising pre-surgical physiological state. Investigation of optimal exercise prescriptions, including intensity, duration, and modality, based on molecular response patterns could improve prehabilitation effectiveness.

Conclusion

This study presents novel molecular evidence in skeletal muscle tissue that prehabilitation preserves mitochondrial function and modulates inflammatory responses in cancer patients undergoing major surgery. The maintenance of electron transport chain complexes in the prehabilitation group offer biological explanations for the clinical benefits reported in previous trials (reduced length

of stay, risk of complications). Whilst the small sample size and lack of significant correlations with functional outcomes limit definitive conclusions, these findings represent an important step toward understanding prehabilitation mechanisms. The success of the remote supervision model and the feasibility of obtaining meaningful molecular data support the continued development of prehabilitation programs across all centres, regardless of available resources.

These findings contribute to the growing evidence base supporting prehabilitation as a valuable component of cancer care.

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